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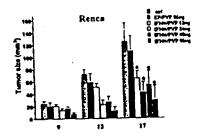
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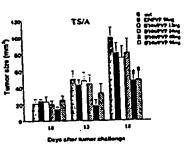
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#### (57) Abstract

The present invention relates to gene delivery and gene therapy, and provides novel nucleic acid constructs for expression of interferon alpha in a mammal, formulations for delivery that incorporate a nucleic acid construct for expression, and methods for preparing and using such constructs and formulations. In particular, this invention relates to plasmid constructs for delivery of therapeutic interferon alpha encoding nucleic acids to cells in order to modulate tumor activity, methods of using those constructs (including combination therapy with other agents, such as cytokines, preferably IL-12), as well as methods for preparing such constructs.





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#### DESCRIPTION

# Interferon Alpha Plasmids And Delivery Systems, And Methods Of Making And Using The Same

#### Related Applications

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This application relates to U.S. patent application Serial No. 08/949,160, filed October 10, 1997 and International patent application No. PCT/US97/18779, filed October 10, 1997, (Lyon & Lyon Docket Nos. 226/285 US and PCT, respectively), both of which are related to U.S. patent application Serial No. 60/028,676, filed October 18, 1996, (Lyon & Lyon Docket No. 222/086 US), all three of which are entitled "IL-12 GENE EXPRESSION AND DELIVERY SYSTEMS AND USES" (by Nordstrom et al.).

This application is also related to U.S. patent application Serial No. 08/798,974, filed February 11, 1997, (Lyon & Lyon Docket No. 224/084 US) and International patent application No. PCT/US95/17038, filed December 28, 1995, (Lyon & Lyon Docket No. 210/190 PCT), both of which are related to U.S. patent application Serial No. 08/372,213, filed January 13, 1995, (Lyon & Lyon Docket No. 210/190 US), all three of which are entitled "FORMULATED NUCLEIC ACID COMPOSITIONS AND METHODS OF ADMINISTERING THE SAME FOR GENE THERAPY" (by Mumper Rolland).

Each of the above-mentioned applications are incorporated herein by reference in their entirety, including any drawings.

#### Field Of The Invention

The present invention relates to gene delivery and gene therapy, and provides novel nucleic acid constructs for expression of interferon alpha in a mammal, formulations for delivery that incorporate a nucleic acid construct for expression, and methods for preparing and using such constructs and formulations. In particular, this invention

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relates to plasmid constructs for delivery of therapeutic interferon alpha encoding nucleic acids to cells in order to modulate tumor activity, methods of using those constructs (including combination therapy with other agents, such as cytokines, preferably IL-12), as well as methods preparing such constructs.

#### Background Of The Invention

The following discussion of the background of invention is merely provided to aid the understanding the invention and is not admitted to describe or constitute prior art to the present invention.

Plasmids are an important element in genetic engineering and gene therapy. Plasmids are usually circular DNA molecules that can be introduced into bacterial cells by transformation which replicate autonomously in the cell. 15 Plasmids typically allow for the amplification of cloned Some plasmids are present in 20 to 50 copies during cell growth, and after the arrest of protein synthesis, as many as 1000 copies per cell of a plasmid can be generated. Suzuki et al., Genetic Analysis, p. 404, 1989.

Current non-viral approaches to human gene therapy require that a potential therapeutic gene be cloned into plasmids. Large quantities of a bacterial host harboring the plasmid may be fermented and the plasmid DNA may be 25 purified for subsequent use. Current human clinical trials plasmids utilize this approach. Recombinant Advisory Committee Data Management Report, December, 1994, Human Gene Therapy 6:535-548. Studies normally focus on the therapeutic gene and the elements that control its 30 expression in the patient when designing and constructing gene therapy plasmids. Generally, therapeutic genes and regulatory elements are simply inserted into existing cloning vectors that are convenient and readily available.

Plasmid design and construction utilizes several key 35 factors. First, plasmid replication origins

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plasmid copy number, which affects production yields. Plasmids that replicate to higher copy number can increase plasmid yield from a given volume of culture, but excessive copy number can be deleterious to the bacteria and lead to undesirable effects (Fitzwater, et al., Embo J. 7:3289-3297 (1988); Uhlin, et al., Mol. Gen. Genet. 165:167-179 (1979)). Artificially constructed plasmids are sometimes unstably maintained, leading to accumulation of plasmid-free cells and reduced production yields.

To overcome this problem of plasmid-free cells, genes 10 that code for antibiotic resistance phenotype are included on the plasmid and antibiotics are added to kill or inhibit plasmid-free cells. Most general purpose cloning vectors contain ampicillin resistance ( $\beta$ -lactamase, or bla) genes. Use of ampicillin can be problematic because of 15 potential for residual antibiotic in the purified DNA, which can cause an allergic reaction in a treated patient. addition,  $\beta$ -lactam antibiotics are clinically important for When plasmids containing antibiotic disease treatment. resistance genes are used, the potential exists for the 20 transfer of antibiotic resistance genes to a potential pathogen.

Other studies have used the *neo* gene which is derived from the bacterial transposon Tn5. The *neo* gene encodes resistance to kanamycin and neomycin (Smith, *Vaccine* 12:1515-1519 (1994)). This gene has been used in a number of gene therapy studies, including several human clinical trials (Recombinant DNA Advisory Committee Data Management Report, December, 1994, *Human Gene Therapy* 6:535-548). Due to the mechanism by which resistance is imparted, residual antibiotic and transmission of the gene to potential pathogens may be less of a problem than for  $\beta$ -lactams.

In addition to elements that affect the behavior of the plasmid within the host bacteria, such as  $E.\ coli$ , plasmid vectors have also been shown to affect transfection and expression in eukaryotic cells. Certain plasmid sequences

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have been shown to reduce expression of eukaryotic genes in eukaryotic cells when carried in cis (Peterson, et al., Mol. Cell. Biol. 7:1563-1567 (1987); Yoder et al., Mol. Cell. Biol. 3:956-959 (1983); Lusky et al., Nature 293:79-81

- 5 (1981); and Leite, et al., Gene 82:351-356 (1989)). Plasmid sequences also have been shown to fortuitously contain binding sites for transcriptional control proteins (Ghersa, et al., Gene 151:331-332 (1994); Tully et al., Biochem. Biophys. Res. Comm. 144:1-10 (1987); and Kushner, et al.,
- Mol. Endocrinol. 8:405-407 (1994)). This can cause inappropriate levels of gene expression in treated patients.

Interferon alpha is a gene product that has been proposed for use, either alone or in combination with other agents, in different delivery systems for the treatment of 15 certain diseases, including particular International cancers. patent publication WO/97/00085, January 3, 1997, proposes ex vivo transfection of tumor published cells with interferon alpha and another immomodulatory molecule, such as IL-12. None of the previously proposed treatments have proven entirely satisfactory, due in part to 20 the high cost and technical difficulty involved in ex vivo approaches. Thus there still remains a need in the art for improved plasmids encoding interferon alpha as well as improved treatment protocols and technologies.

#### 25 Summary

The present invention relates to gene delivery and gene therapy, and provides novel nucleic acid constructs for expression of interferon alpha in a mammal, formulations for delivery that incorporate a nucleic acid construct for expression, and methods for preparing and using such constructs and formulations. In particular, this invention relates to plasmid constructs for delivery of therapeutic interferon alpha encoding nucleic acids to cells in order to modulate tumor activity, methods of using those constructs (including combination therapy with other agents, such as

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cytokines, preferably IL-12), as well as methods for preparing such constructs. The pharmaceutical acceptable, cost effective and highly efficient delivery system presented herein represents an unanticipated improvement over the art.

Thus, in a first aspect, the invention features a plasmid that contains a CMV promoter and optionally a synthetic 5' intron transcriptionally linked with an interferon alpha coding sequence, and a 3'-untranslated region (UTR). Preferably the 3' UTR is a 3' growth hormone UTR.

As used herein, the term "plasmid" refers to a construct made up of genetic material (i.e., nucleic acids). It includes genetic elements arranged such that an inserted coding sequence can be transcribed in eukaryotic cells. Also, while the plasmid may include a sequence from a viral nucleic acid, such viral sequence does not cause the incorporation of the plasmid into a viral particle, and the plasmid is therefore a non-viral vector. Preferably a plasmid is a closed circular DNA molecule.

"Cytomegalovirus promoter" refers to one or more sequences from a cytomegalovirus which are functional in eukaryotic cells as a transcriptional promoter and an upstream enhancer sequence. The enhancer sequence allows transcription to occur at a higher frequency from the associated promoter.

In this context, "transcriptionally linked" means that in a system suitable for transcription, transcription will initiate under the direction of the control sequence(s) and proceed through sequences which are transcriptionally linked with that control sequence(s). Preferably no mutation is created in the resulting transcript, which would alter the resulting translation product.

The term "coding region" or "coding sequence" refers to a nucleic acid sequence which encodes a particular gene product for which expression is desired, according to the

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normal base pairing and codon usage relationships. Thus, the coding sequence must be placed in such relationship to transcriptional control sequences (possibly including control elements and translational initiation and termination codons) that a proper length transcript will be produced and will result in translation in the appropriate reading frame to produce a functional desired product.

In a preferred embodiment the interferon alpha coding sequence is for human interferon alpha and preferably is a synthetic sequence having optimal codon usage, such as the nucleotide sequence of SEQ ID NO:11 or semi-optimal codon usage, such as the nucleotide sequence of SEQ ID NO:12.

A particular example of coding regions suitable for use in the plasmids of this invention are the natural sequences 15 coding for human interferon alpha. Thus, in a preferred embodiment coding region has a nucleotide sequence which is the same as SEQ ID NO:10, which is the natural nucleotide sequence encoding human interferon alpha. However, it may be preferable to have an interferon alpha coding sequence which is a synthetic coding sequence. 20 In a preferred embodiment, the interferon alpha coding sequence synthetic sequence utilizing optimal or semi-optimal codon usage, preferably the sequence shown in SEQ ID NO:11 or SEQ

25 a "sequence coding for the human interferon alpha" or "a human interferon alpha coding sequence" is a nucleic acid sequence which encodes the amino acid sequence of human interferon alpha, based on the normal base pairing translational codon usage relationships. 30 preferable that the coding sequence encode the exact, full amino acid sequence of natural human interferon, but this is not essential. The encoded polypeptide may differ from natural human interferon alpha, so long as the polypeptide interferon alpha activity, preferably polypeptide is at least 50%, 75%, 90%, or 97% as active as 35 natural human interferon alpha, and more preferably fully as

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active as natural human interferon alpha. Thus, the polypeptide encoded by the interferon alpha coding sequence may differ from a natural human interferon alpha polypeptide by a small amount, preferably less than a 15%, 10%, 5%, or 1% change. Such a change may be of one of more different types, such as deletion, addition, or substitution of one or more amino acids.

The term "transcriptional control sequence" refers to sequences which control the rate of transcription of a transcriptionally linked coding region. Thus, the term can promoters, operators, as elements such transcription unit, the particular enhancers. For а transcriptional control sequences will include at least a promoter sequence.

A "growth hormone 3' untranslated region" is a sequence 15 of the region encoding located downstream (i.e., 3') material polypeptide and including at least part of the sequence of the natural 3' UTR/poly(a) signal from a growth hormone gene, preferably the human growth hormone gene. This region is generally transcribed but not translated. 20 generally is it eukaryotic cells in expression preferable to include sequence which signals the addition of a poly-A tail. As with other synthetic genetic elements a synthetic 3' UTR/poly(A) signal has a sequence which differs from naturally-occurring UTR elements. 25

The sequence may be modified, for example by the deletion of ALU repeat sequences. Deletion of such ALU repeat sequences acts to reduce the possibility of homologous recombination between the expression cassette and genomic material in a transfected cell.

The plasmid preferably includes a promoter, a TATA box, a Cap site and a first intron and intron/exon boundary in appropriate relationship for expression of the coding sequence. The plasmid may also include a 5' mRNA leader sequence inserted between the promoter and the coding sequence and/or an intron/5' UTR from a chicken skeletal  $\alpha$ -

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actin gene. Also, the plasmid may have a nucleotide sequence which is the same as the nucleotide sequence of plasmid pIF0921, as shown in Figure 5.

The plasmid may also include: (a) a first transcription unit containing a first transcriptional control sequence 5 transcriptionally linked with а first 5'-untranslated region, a first intron, a first coding sequence, and a first 3'-untranslated region/poly(A) signal, wherein the first intron is between the control sequence and the first coding sequence; and (b) a second transcription unit containing a 10 second transcriptional control sequence transcriptionally linked with a second 5'-untranslated region, intron, second coding sequence, and a second untranslated region/poly(A) signal, wherein intron is between the control sequence and the second coding 15 sequence; wherein the first and second coding sequences contain a sequence having the sequence of SEQ ID NO:2, 3, 4 or 25 coding for a human IL-12 p40 subunit, and a sequence having the sequence of SEQ ID NO:6, 7, 8 or 24 coding for a 20 human IL-12 p35 subunit.

The term "transcription unit" or "expression cassette" refers to a nucleotide sequence which contains at least one coding sequence along with sequence elements which direct initiation and termination of transcription. transcription unit may however include additional sequences, 25 which may include sequences involved in post-transcriptional or post-translational processes. In preferred embodiments, the first transcriptional control sequence or the second transcriptional control sequence contain 30 cytomegalovirus promoter sequences. one The first and second transcriptional control sequences can be the different. same

A "5' untranslated region" or "5' UTR" refers to a sequence located 3' to promoter region and 5' of the downstream coding region. Thus, such a sequence, while transcribed, is upstream of the translation initiation codon

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and therefore is not translated into a portion of the polypeptide product.

For the plasmids described herein, one or more of a promoter, untranslated region (5' UTR), UTR/poly(A) signal, and introns may be a synthetic sequence. In this context the term "synthetic" means that the sequence is not provided directly by the sequence of a naturally occurring genetic element of that type but rather is an artificially created sequence (i.e., created by a person by molecular biological methods). While one or more portions of such a synthetic sequence may be the same as portions of naturally occurring sequences, the full sequence over the specified genetic element is different from a naturally occurring genetic element of that type. The use of such elements synthetic genetic allows the functional characteristics of that element to be appropriately designed for the desired function.

Thus, a "synthetic intron" refers to a sequence which is not a naturally occurring intron sequence but which will be removed from an RNA transcript during normal post transcriptional processing. Such introns can be designed to have a variety of different characteristics, in particular such introns can be designed to have a desired strength of splice site.

A "subunit" of a therapeutic molecule is a polypeptide or RNA molecule which combines with one or more other molecules to form a complex having the relevant pharmacologic activity. Examples of such complexes include homodimers and heterodimers as well as complexes having greater numbers of subunits. A specific example of a heterodimer is IL-12, having the p40 and p35 subunits.

The "p40 subunit" is the larger of the two subunits of the IL-12 heterodimer. Thus, it is capable of association with p35 subunit to form a molecule having activities characteristic of IL-12. Human p40 has the amino acid sequence of SEQ ID NO:1. Those skilled in the art will

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recognize that the molecule may have a number of changes from that sequence, such as deletions, insertions or changes of one or a few amino acids, while still retaining IL-12 activity when associated with p35. Such active altered molecules are also regarded as p40.

Conversely, the "p35 subunit" is the smaller of the two heterodimeric subunits of IL-12. For humans, p35 has the amino acid sequence of SEQ ID NO:5. As for p40, p35 may have a low level of alterations from that sequence while still being regarded as p35.

A particular example of coding regions suitable for use in the plasmids of this invention are the natural sequences coding for the p40 and p35 subunits of human IL-12. Thus, in a preferred embodiment the first and second coding regions are coding regions for those sequences and are preferably in the order p40 then p35 in the 5' to 3' direction.

Thus, a "sequence coding for the p40 subunit of human IL-12" is a nucleic acid sequence which encodes the human p40 subunit as described above, based on the normal base pairing and translational codon usage relationships. The sequence coding for p35 subunit of human IL-12 is similarly defined.

In a preferred embodiment the sequence coding for the p40 subunit of human IL-12 is 5' to the sequence coding for the p35 subunit of human IL-12. Those skilled in the art will appreciate that the interferon alpha, p35 subunit and p40 subunit may all be on a single transcription unit, that all three may be on separate transcription units, or that any two coding sequences may be on one transcription unit and the other coding sequence on another transcription unit.

The plasmid may also contain an intron having variable splicing, a first coding sequence, and a second coding sequence, wherein the first and second coding sequences include a sequence having the sequence of SEQ ID NO:2, 3, 4 or 25 coding for a human IL-12 p40 subunit, and a sequence

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having the sequence of SEQ ID NO:6, 7, 8 or 24 coding for a human IL-12 p35 subunit.

In a preferred embodiment, the plasmid also has: (a) a transcriptional control sequence transcriptionally linked with a first coding sequence and a second coding sequence; (b) a 5'-untranslated region; (c) an intron 5' to the first coding sequence; (d) an alternative splice site 3' to the first coding sequence and 5' to the second coding sequence; 3'-untranslated region/poly(A) The signal. (e) preferably includes a transcriptional control sequence cytomegalovirus promoter sequence.

In a preferred embodiment, the plasmid also has: (a) a transcriptional control sequence transcriptionally linked with a first coding sequence, an IRES sequence, a second 3'-untranslated region/poly(A) а and coding sequence, signal, wherein the IRES sequence is between the first coding sequence and the second coding sequence; and (b) an intron between the promoter and the first coding sequence; wherein the first and second coding sequences include a sequence having the sequence of SEQ ID NO:2, 3, 4 or 25 coding for a human IL-12 p40 subunit, and a sequence having the sequence of SEQ ID NO:6, 7, 8 or 24 coding for a human The transcriptional control sequence IL-12 p35 subunit. preferably includes a cytomegalovirus promoter sequence and the IRES sequence preferably is from an encephalomyocarditis virus.

For delivery of coding sequences for gene expression, it is generally useful to provide a delivery composition or delivery system which includes one or more other components in addition to the nucleic acid sequences. Such a composition can, for example, aid in maintaining the integrity of the DNA and/or in enhancing cellular uptake of the DNA and/or by acting as an immunogenic enhancer, such as by the non-DNA components having an immuno-stimulatory effect of their own.

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Thus, in another aspect, the invention features a composition containing a plasmid as described above and a protective, interactive non-condensing compound (PINC).

The PINC enhances the delivery of the nucleic acid molecule to mammalian cells in vivo, and preferably the 5 nucleic acid molecule includes a coding sequence for a gene product to be expressed in the cell. In many cases, the relevant gene product is a polypeptide Preferably the PINC is used under conditions so that the PINC does not form a gel, or so that no gel form is present 10 at the time of administration at about  $30-40\Box C$ . these compositions, the PINC is present at a concentration of 30% (w/v) or less. In certain preferred embodiments, the PINC concentration is still less, for example, 20% or less, 10% or less, 5% or less, or 1% or less. 15 compositions differ in compound concentration and functional effect from uses of these or similar compounds in which the compounds are used at higher concentrations, for example in the ethylene glycol mediated transfection 20 protoplasts, or the formation of gels for drug or nucleic acid delivery. In general, the PINCs are not in gel form in the conditions in which they are used as PINCs, though the compounds may form gels under conditions.

25 In connection with the compounds and compositions of this invention, the term "protects" or "protective" refers to an effect of the interaction between such a compound and a nucleic acid such that the rate of degradation of the nucleic acid is decreased in a particular environment. 30 degradation may be due to a variety of different factors, which specifically include the enzymatic action nuclease. The protective action may be different ways, for example, by exclusion of the nuclease provided molecules or by exclusion of water.

Some compounds which protect a nucleic acid and/or prolong the bioavailability of a nucleic acid may also

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interact or with associate the nucleic acid by intermolecular forces and/or valence bonds such as: Waals forces, ion-dipole interactions, ion-induced dipole interactions, hydrogen bonds, or ionic bonds. interactions may the following functions: serve (1)Stereoselectively protect nucleic acids from nucleases by shielding; (2) facilitate the cellular uptake of nucleic acid by "piggyback endocytosis". Piggyback endocytosis is the cellular uptake of a drug or other molecule complexed to a carrier that may be taken up by endocytosis. CV Uglea and Dumitriu-Medvichi, Medical Applications of Synthetic Oligomers, In: Polymeric Biomaterials, Severian Dumitriu ed., Marcel Dekker, Inc., 1993, incorporated herein by reference.

15 To achieve the desired effects set forth is desirable, but not necessary, that the compounds which protect the nucleic acid and/or prolong the bioavailability of a nucleic acid have amphiphilic properties; that is, have both hydrophilic and hydrophobic regions. The hydrophilic 20 region of the compounds may associate with the largely ionic and hydrophilic regions of the nucleic acid, while the hydrophobic region of the compounds may act to retard diffusion of nucleic acid and to protect nucleic acid from nucleases.

Additionally, the hydrophobic region may specifically interact with cell membranes, possibly facilitating endocytosis of the compound and thereby also of nucleic acid associated with the compound. This process may increase the pericellular concentration of nucleic acid.

Agents which may have amphiphilic properties and are generally regarded as being pharmaceutically acceptable are the following: polyvinylpyrrolidones; polyvinylalcohols; polyvinylacetates; propylene glycol; polyethylene glycols; poloxamers (Pluronics); poloxamines (Tetronics); ethylene vinyl acetates; methylcelluloses, hydroxypropylcelluloses, hydroxypropylmethylcelluloses; heteropolysaccharides

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(pectins); chitosans; phosphatidylcholines (lecithins); miglyols; polylactic acid; polyhydroxybutyric acid; xanthan Also, copolymer systems such as polyethylene glycolpolylactic acid (PEG-PLA), polyethylene polyhydroxybutyric acid (PEG-PHB), polyvinylpyrrolidonepolyvinylalcohol (PVP-PVA), and derivatized copolymers such as copolymers of N-vinyl purine (or pyrimidine) derivatives and N-vinylpyrrolidone. However, not all of the above compounds are protective, interactive, non-condensing compounds as described below.

In connection with the protective, interactive, non-condensing compounds for these compositions, the term "non-condensing" means that an associated nucleic acid is not condensed or collapsed by the interaction with the PINC at the concentrations used in the compositions. Thus, the PINCs differ in type and/or use concentration from such condensing polymers. Examples of commonly used condensing polymers include polylysine, and cascade polymers (spherical polycations).

20 Also connection with such compounds an associated nucleic acid molecule, the term "enhances the delivery" means that at least in conditions such that the amounts of PINC and nucleic acid is optimized, a greater biological effect is obtained than with the delivery of nucleic acid in saline. Thus, in cases where the expression 25 of a gene product encoded by the nucleic acid is desired, the level of expression obtained with the PINC: nucleic acid composition is greater than the expression obtained with the same quantity of nucleic acid in saline for delivery by a method appropriate for the particular PINC/coding sequence 30 combination.

In preferred embodiments of the above compositions, the PINC is polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), a PVP-PVA co-polymer, N-methyl-2-pyrrolidone (NM2P), ethylene glycol, or propylene glycol. In compositions in which a Poloxamer (Pluronics) is used, the nucleic acid is

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preferably not a viral vector, i.e., the nucleic acid is a non-viral vector.

In other preferred embodiments, the PINC is bound with a targeting ligand. Such targeting ligands can be of a variety of different types, including but not limited to galactosyl, residues, fucosal residues, mannosyl residues, carntitine derivatives, monoclonal antibodies, polyclonal antibodies, peptide ligands, and DNA-binding proteins. The targeting ligands may bind with receptors on cells such as antigen-presenting cells, hepatocytes, myocytes, epithelial cells, endothelial cells, and cancer cells.

In connection with the association of a targeting ligand and a PINC, the term "bound with" means that the parts have an interaction with each other such that the physical association is thermodynamically favored, representing at least a local minimum in the free energy function for that association. Such interaction may involve covalent binding, or non-covalent interactions such ionic, hydrogen bonding, van der Waals interactions. hydrophobic interactions, and combinations of such interactions.

While the targeting ligand may be of various types, in one embodiment the ligand is an antibody. Both monoclonal antibodies and polyclonal antibodies may be utilized.

The nucleic acid may also be present in various forms. Preferably the nucleic acid is not associated with a compounds(s) which alter the physical form, however, in other embodiments the nucleic acid is condensed (such as with a condensing polymer), formulated with cationic lipids, formulated with peptides, or formulated with cationic polymers.

In preferred embodiments, the protective, interactive non-condensing compound is polyvinyl pyrrolidone, and/or the plasmid is in a solution having between 0.5% and 50% PVP, more preferably about 5% PVP. The DNA preferably is at least about 80% supercoiled, more preferably at least about

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90% supercoiled, and most preferably at least about 95% supercoiled.

In another aspect the invention features a composition containing a protective, interactive non-condensing compound and a plasmid containing an interferon alpha coding sequence.

In yet another aspect, the invention provides a composition containing a plasmid of the invention (or a plasmid containing an interferon alpha coding sequence) and a cationic lipid with a neutral co-lipid.

Preferably the cationic lipid is DOTMA and the neutral co-lipid is cholesterol (chol). DOTMA is 1,2-di-O-octadecenyl-3-trimethylammonium propane, which is described and discussed in Eppstein et al., U.S. Patent 4,897,355, issued January 30, 1990, which is incorporated herein by reference. However, other lipids and lipid combinations may be used in other embodiments. A variety of such lipids are described in Gao & Huang, 1995, Gene Therapy 2:710-722, which is hereby incorporated by reference.

As the charge ratio of the cationic lipid and the DNA is also a significant factor, in preferred embodiments the DNA and the cationic lipid are present is such amounts that the negative to positive charge ratio is about 1:3. While preferable, it is not necessary that the ratio be 1:3.

Thus, preferably the charge ratio for the compositions is between about 1:1 and 1:10, more preferably between about 1:2 and 1:5.

The term "cationic lipid" refers to a lipid which has a net positive charge at physiological pH, and preferably carries no negative charges at such pH. An example of such a lipid is DOTMA. Similarly, "neutral co-lipid" refers to a lipid which has is usually uncharged at physiological pH. An example of such a lipid is cholesterol.

Thus, "negative to positive charge ratio" for the DNA and cationic lipid refers to the ratio between the net

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negative charges on the DNA compared to the net positive charges on the cationic lipid.

form of the the DNA affects the expression efficiency, the DNA preferably is at least about supercoiled, more preferably at least about 90% supercoiled, and most preferably at least about 95% supercoiled. composition preferably includes an isotonic carbohydrate solution, such as an isotonic carbohydrate solution that consists essentially of about 10% lactose. In preferred embodiments, the composition the cationic lipid and the neutral co-lipid are prepared as a liposome having extrusion size of about 800 nanometers. Preferably the liposomes are prepared to have an average diameter between about 20 and 800 nm, more preferably between about 50 and 400 nm, still more preferably between about 75 and 200 nm, and most preferably about 100 nm. Microfluidization is the preferred method of preparation of the liposomes.

In another aspect the invention features a composition containing: (a) a first component having a plasmid including an interferon alpha coding sequence and a cationic lipid with a neutral co-lipid, wherein the cationic lipid is DOTMA and the neutral co-lipid is cholesterol, wherein the DNA in the plasmid and the cationic lipid are present in amounts such that the negative to positive charge ratio is about 1:3; and (b) a second component including a protective, interactive non-condensing compound, wherein the first component is present within the second component.

In another aspect, the invention provides a composition having a protective, interactive non-condensing compound, a first plasmid including an interferon alpha coding sequence, and one or more other plasmids independently having an IL-12 p35 or IL-12 p40 subunit coding sequence.

In another aspect, the invention features a method for making any of the plasmids described above by inserting a CMV promoter transcriptionally linked with an interferon

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alpha coding sequence, and a growth hormone 3'-untranslated region into a plasmid.

The invention also provides methods of making the compositions described above. The method may involve: (a) preparing a DNA molecule having a transcriptional unit, wherein the transcriptional unit contains an interferon alpha coding sequence; (b) preparing a protective, interactive non-condensing compound; and (c) combining the protective, interactive non-condensing compound with the DNA in conditions such that a composition capable of delivering a therapeutically effective amount of an interferon alpha coding sequence to a mammal is formed.

Preferably, the DNA molecule is a plasmid, wherein the plasmid includes a CMV promoter transcriptionally linked with an interferon alpha coding sequence, and a human growth hormone 3'-untranslated region/poly(A) signal.

The method may involve the steps of: (a) preparing a DNA having an interferon alpha coding sequence; (b) preparing a mixture of a cationic lipid and a neutral colipid, wherein the cationic lipid is DOTMA and the neutral colipid is cholesterol; and (c) combining the mixture with the DNA in amounts such that the cationic lipid and the DNA are present in a negative to positive charge ratio of about 1:3.

25 In another embodiment, the method involves the steps preparing a first component having a plasmid containing an interferon alpha coding sequence cationic lipid with a neutral co-lipid, wherein the cationic lipid is DOTMA and the neutral co-lipid is cholesterol, wherein the DNA in the plasmid and the cationic lipid are 30 present in amounts such that the negative to positive charge ratio is about 1:3; (b) preparing a second component having a protective, interactive non-condensing compound; and (c) combining the first and second components such that the resulting composition includes the first component within 35 the second component.

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In another embodiment, the method involves the steps of: (a) preparing a protective, interactive non-condensing compound, (b) preparing a first plasmid having an interferon alpha coding sequence, (c) preparing one or more other plasmids independently having an IL-12 p35 or IL-12 p40 subunit coding sequence, and (d) combining the protective, interactive non-condensing compound, the plasmid having the interferon alpha coding sequence and the other plasmids.

In another aspect, the invention provides a method for treatment of a mammalian condition or disease, by administering to a mammal suffering from the condition or disease a therapeutically effective amount of a plasmid as described herein.

A "therapeutically effective amount" of a composition is an amount which is sufficient to cause at least temporary 15 relief or improvement in a symptom or indication of a disease or condition. Thus, the amount is also sufficient to cause a pharmacological effect. The amount of the composition need not cause permanent improvement 20 improvement of all symptoms or indications. Α therapeutically effective amount of a cancer therapeutic would be one that reduces overall tumor burden in the case of metastatic disease (i.e., the number of metasteses or their size) or one that reduces the mass of the tumor in 25 localized cancers.

The condition or disease preferably is a cancer, such epithelial glandular cancer, including adenoma adenocarcinoma; squamous and transitional cancer, including papilloma, squamous cell and transitional carcinoma; connective tissue cancer, including tissue type positive, sarcoma and other (oma's); hematopoietic lymphoreticular cancer, including lymphoma, leukemia and Hodgkin's disease; neural tissue cancer, including neuroma, sarcoma, neurofibroma and blastoma; mixed tissues of origin including teratoma and teratocarcinoma. cancer, cancerous conditions that are applicable to treatment

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include cancer of any of the following: adrenal gland, anus, bile duct, bladder, brain tumors: adult, breast, cancer of an unknown primary site, carcinoids of the gastrointestinal cervix, childhood cancers, colon and esophagus, gall bladder, head and neck, islet cell and other pancreatic carcinomas, Kaposi's sarcoma, kidney, leukemia, lung: non-small cell, lung: small cell, lymphoma: AIDS-associated, lymphoma: Hodgkin's disease, Lymphomas: non-Hodgkin's disease, melanoma, mesothelioma, cancer, multiple myeloma, ovary, ovarian germ cell tumors, pancreas, parathyroid, penis, pituitary, prostate, sarcomas of bone and soft tissue, skin, small intestine, testis, thymus, thyroid, trophoblastic disease, uterus: endometrial carcinoma, uterus: uterine sarcomas, vagina, or The composition preferably is administered by injection, although the method may also be performed vivo.

In another aspect, the invention provides a method for transfection (i.e., the delivery and expression of a gene to cells) of a cell in situ, by contacting the cell with a plasmid described herein for sufficient time to transfect the cell. Transfection of the cell preferably is performed in vivo and the contacting preferably is performed in the presence of about 5% PVP solution.

In another aspect, the invention features a method for delivery and expression of an interferon alpha gene in a plurality of cells, by: (a) transfecting the plurality of cells with a plasmid or composition of the invention; and (b) incubating the plurality of cells under conditions allowing expression of a nucleic acid sequence in the vector, wherein the nucleic acid sequence encodes interferon alpha.

In preferred embodiments, the interferon alpha is human interferon alpha and the cells are human cells and/or the contacting is performed in the presence of an about 5% PVP solution.

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In another aspect, the invention features a method for treating a disease or condition, by transfecting a cell in situ with a plasmid or composition of the invention. The disease or condition can be a localized disease or condition or a systemic disease or condition.

In another aspect, the invention features a cell transfected with a plasmid or composition of the invention.

In yet another aspect, the invention features a method for treatment of a mammalian condition or disease, by administering to a mammal suffering from the condition or disease a therapeutically effective amount of a composition described herein.

As the compositions are useful for delivery of a nucleic acid molecule to cells in vivo, in a related aspect the invention provides a composition at an in vivo site of administration. In particular this includes at an in vivo site in a mammal.

In preferred embodiments the nucleic acid molecule includes a sequence encoding a gene product. Also in preferred embodiments, the site of administration is in an interstitial space or a tissue of an animal, particularly of a mammal.

The invention also provides methods for using the above compositions. Therefore, in further related aspects, methods of administering the compositions are provided in which the composition is introduced into a mammal, preferably into a tissue or an interstitial space.

Various methods of delivery may be utilized, such as are known in the art, but in preferred embodiments, the composition is introduced into the tissue or interstitial space by injection. The compositions may also be delivered to a variety of different tissues, but in preferred embodiments the tissue is muscle or tumor.

In another related aspect, the invention provides 35 methods for treating a mammalian condition or disease by administering a therapeutically effective amount of a

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composition as described above. In preferred embodiments, the disease or condition is a cancer.

The summary of the invention described above is non-limiting and other features and advantages of the invention will be apparent from the following detailed description of the preferred embodiments, as well as from the claims.

#### Brief Description Of The Drawings

Figure 1 shows the effects of interferon alpha in two cancer models.

10 Figure 2 shows a plasmid map and sequence (SEQ ID NO:18) for an exemplary IL-12 plasmid of the present invention.

Figure 3 shows optimal codon usage for highly expressed human genes.  $\ensuremath{\mathsf{S}}$ 

Figure 4 shows a plasmid map and sequence (SEQ ID NO:19) for plasmid pIF0836, an exemplary interferon alpha plasmid of the present invention.

Figure 5 shows a plasmid map and sequence (SEQ ID NO:20) for pIN096, an exemplary IL-12 plasmid that can be used with the present invention.

Figure 6 shows the nucleic acid sequence (SEQ ID NO:21) of plasmid pIF0921, an exemplary interferon alpha plasmid of the present invention.

Figures 7A and 7B show a plasmid map and sequence (SEQ 25 ID NO:22) for plasmid pIF0921.

Figure 8 shows an outline of a strategy that can be used to synthesize a pIF0921 plasmid.

Figure 9 shows interferon alpha and IL-12 gene medicine (combination therapy) in Renca model.

### 30 Detailed Description Of The Preferred Embodiments

The present invention relates to gene delivery and gene therapy, and provides novel nucleic acid constructs for expression of interferon alpha in a mammal, formulations for delivery that incorporate a nucleic acid construct for

expression, and methods for preparing and using constructs and formulations. In particular, this invention relates to plasmid constructs for delivery of therapeutic interferon alpha encoding nucleic acids to cells in order to modulate tumor activity, methods of using those constructs (including combination therapy with other agents, such as preferably IL-12), cytokines, as well as methods for preparing such constructs.

#### I. General

As described, this invention concerns expression systems for the delivery and expression of interferon alpha coding sequences in mammalian cells, and formulations and methods for delivering such expression systems or other expression systems to a mammal.

Therefore, particular genetic constructs are described which includes nucleotide sequences coding for interferon alpha, preferably human interferon alpha. Such a construct can beneficially be formulated and administered as described herein, utilizing the expression systems of this invention.

To allow convenient production of such plasmids, it is generally preferable that the plasmid be capable of replication in a cell to high copy number. Generally such production is carried out in prokaryotic cells, particularly including Esherichia coli (E.coli) cells. Thus, the plasmid preferably contains a replication origin functional in a prokaryotic cell, and preferably the replication origin is one which will direct replication to a high copy number.

It is also possible to utilize synthetic genetic elements in the plasmid constructs.

As described below, these elements affect posttranscriptional processing in eukaryotic systems. Thus, the use of synthetic sequences allows the design of processing characteristics as desired for the particular application. Commonly, the elements will be designed to provide rapid and accurate processing.

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For delivery of DNA encoding a desired expression product to a mammalian system, it is usually preferable to utilize a delivery system. Such a system can provide multiple benefits, notably providing stabilization to protect the integrity of the DNA, as well as assisting in cellular uptake.

In addition, the non-DNA components of the formulation may contribute to an immune system enhancement As a result, components of a delivery system activation. selected in conjunction with a particular gene can be product to enhance or minimize the immuno-stimulatory effect.

The plasmids may also include elements for expression of IL-12 or one or more subunits thereof. Similarly, the treatment may involve administration of an interferon alpha coding sequence and one or more IL-12 coding sequences whether on a single plasmid or on separate plasmids. Such plasmids may be incorporated into compositions for delivery with a protective, interactive non-condensing compound, a cationic lipid and neutral co-lipid, or both.

While these are specific effective examples, other components may be utilized in formulations containing the interferon alpha expression vectors of the present invention to provide effective delivery and expression of interferon alpha or with other coding sequences for which manipulation of the activation of immune system components is desirable.

The selection of delivery system components and preparation methods in conjunction with the selection of coding sequences provides the ability to balance the specific effects of the encoded gene products and the immune system effects of the overall delivery system composition. This capacity to control the biological effects of delivery system formulation administration represents an aspect of the invention in addition to the interferon alpha encoding constructs and specific formulations of delivery systems. Co-selection of the encoded gene product and the delivery

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system components and parameters provides enhanced desired effects rather than merely providing high level gene expression. In particular, such enhancement is described below for the antitumor effects of the exemplary PVP containing compositions.

For systems in which IL-12 is also administered, the antitumor effect can be greater than merely additive (i.e., greater than merely the sum of the antitumor effects of interferon alpha alone and IL-12 alone). Enhancement of immuno-stimulatory effects is also desirable in other contexts, for example, for vaccine applications.

In contrast, for certain applications, it is preferable to select a delivery systems which minimizes the immune system effects. For example, it is often preferred that the immune system activation be minimized for compositions to be delivered to the lung in order to minimize lung tissue swelling.

A useful approach for selecting the delivery system components and preparation techniques for a particular coding sequence can proceed as follows, but is not limited to these steps or step order.

- Select a particular genetic construct which provides appropriate expression in vitro.
- Select delivery system components based on desired immunostimulatory effects and/or on in vivo physiological effect. Such effects can be tested or verified in in vivo model systems.
- 3. Select the other delivery system parameters consistent with the desired immuno-stimulatory effects and/or consistent with enhancing the desired in vivo physiological effect. Such parameters can, for example, include the amount and ratio of DNA to one or more other composition components, the relative amounts of non-DNA composition components, the size of delivery system formulation particles, the percent

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supercoiled DNA for circular dsDNA vectors, and the specific method of preparation of delivery system formulation particles. The particular parameters relevant for specific types of formulations will be apparent or readily determined by testing.

The description below illustrates the selection of components and parameters in the context of interferon alpha encoding constructs. However, it should be recognized that the approach is applicable to constructs containing a variety of other coding sequences.

## II. Plasmid Construct Expression Systems

## A. Plasmid Design and Construction

For the methods and constructs of this invention, a number of different plasmids were constructed which are useful for delivery and expression of sequences encoding interferon alpha. Thus, these plasmids contain coding regions for interferon alpha, along with genetic elements necessary or useful for expression of those coding regions.

While these embodiments utilized interferon alpha cDNA clones or partial genomic sequences from a particular source, those skilled in the art could readily obtain interferon alpha coding sequences from other sources, or obtain a coding sequence by identifying a cDNA clone in a library using a probe(s) based on the published interferon alpha coding and/or flanking sequences. This also applies to the IL-12 coding sequences utilized in the embodiments described herein.

Coding sequences for interferon alpha were incorporated into an expression plasmid that contains eukaryotic and bacterial genetic elements. Eukaryotic genetic elements include the CMV immediate early promoter and 5' UTR, and a human growth hormone 3' UTR/poly(a) signal, which influence gene expression by controlling the accuracy and efficiency of RNA processing, mRNA stability, and translation.

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The human growth hormone 3' UTR is from a human growth hormone gene, and preferably includes a poly(a) signal. This sequence can be linked immediately following the natural translation termination codon for a cDNA sequence, genomic sequence, modified genomic sequence, or synthetic sequence coding for interferon alpha.

An example of a human growth hormone 3' UTR/poly(a) signal is shown by the human growth hormone 3' UTR (nucleotides 1 - 100) and 3' flanking sequence (nucleotides 101 - 191; GenBank accession #J03071, HUMGHCSA) below. (SEQ ID NO:13)

1 GGGTGGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGT
Poly (a)signal

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- 51 TGCCACTCCAGTGCCCACCAGCCTTGTCCTAATAAAATTAAGTTGCATCA
- 101 TTTTGTCTGACTAGGTGTCCTTCTATAATATTATGGGGTGGAGGGGGGGTG
- 20 151 GTATGGAGCAAGGGGCAAGTTGGGAAGACAACCTGTAGGGC

The 5' and 3' UTR and flanking regions can be further and more precisely defined by routine methodology, e.g., deletion or mutation analysis or their equivalents., and can be modified to provide other sequences having appropriate transcriptional and translational functions.

1. Construction of plasmid: Plasmid Backbone, human interferon alpha cDNA, Final Construct

A diagrammatic representation of the PCR products and plasmids involved in creation of an exemplary construct is shown below in Figure 8.

Plasmid pIF0921 was constructed from commercially available plasmids, and contains the TN5 gene encoding the kanamycin resistance gene, the pUC origin of replication, the CMV enhancer and promoter to base +112, a synthetic intron called IVS8, the human IFN-a2b gene, and the human growth hormone 3' UTR. The plasmid construction descendancy

for pIL0697 is shown in Figure 8. pIL0697 was cut with BamHI and Xba I and the hIFN-a2b PCR product, which had been amplified from human genomic DNA with BamHI and Xba I ends, was cloned into the pIL0697 backbone in place of the IL-2 coding region. The resulting plasmid was pIF0863. pIF0863 was cut with Nco I and intron IVS8 from pCT0828 was cloned in. The resulting plasmid was pIF0890. pIF0890 was cut with Nde I and Pac I and an additional region of the CMV 5' UTR to base +112 was cloned in from plasmid pLC0888.

#### 10 B. Synthetic Genetic Elements

Ιn some embodiments, some or all of the genetic elements can be synthetic, derived from synthetic oligonucleotides, and thus are not obtained directly from natural genetic sequences. These synthetic elements are appropriate for use in many different expression vectors.

A synthetic intron is designed with splice sites that ensure that RNA splicing is accurate and efficient. A synthetic 3' UTR/poly(A) signal is designed to facilitate mRNA 3' end formation and mRNA stability. A synthetic 5' UTR is designed to facilitate the initiation of translation. The design of exemplary synthetic elements is described in more detail below.

# Summary of Synthetic Element Features Exemplary synthetic 5'UTR, intron, and 3'UTR/poly(A) signal have the general features shown below:

5' UTR Short.

Lack of secondary structure.

Kozak sequence.

Site for intron insertion.

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Intron

5' splice site sequence matches consensus. 5' splice site sequence is exactly complementary to 5' end of U1 snRNA. Branch point sequence matches consensus. Branch point sequence is complementary to U2 snRNA. 3' splice site matches consensus. Polypyrimidine tract is 16 bases in length and contains 7 consecutive T's. (The tract preferably contains at least 5 consecutive T's.) Contains internal restriction enzyme sites.

BbsI cleaves at the 5'ss, EarI

3' UTR/Poly(A) Based on rabbit β-globin 3'
UTR/poly(A) signal.
Consists of two poly(A) signals in tandem.

#### Features of the Synthetic 5'UTR (UT6):

cleaves at the 3'ss.

The 5' untranslated region (5'UTR) influences the translational efficiency of messenger RNA, and is therefore an important determinant of eukaryotic gene expression. The synthetic 5'UTR sequence (UT6) has been designed to maximize the translational efficiency of mRNAs encoded by vectors that express genes of therapeutic interest.

The sequence of the synthetic 5' UTR (UT6) is shown 10 below. The Kozak sequence is in boldface and the initiation codon is double underlined. The location of the intron (between residues 48 and 49) is indicated by the filled

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triangle and the sequences that form the exonic portion of consensus splice sites are single underlined. The restriction sites for HindIII and NcoI are overlined. (SEQ ID NO:14)

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The 5' untranslated region (5' UTR), located between the cap site and initiation codon, is known to influence the 10 efficiency of mRNA translation. Any features that influence the accessibility of the 5' cap structure to initiation factors, the binding and subsequent migration of the 43S preinitiation complex, or the recognition of the initiation codon, will influence mRNA translatability. An efficient 5' 15 UTR is expected to be one that is moderate in length, devoid secondary structure, devoid of upstream initiation and has an AUG within an optimal local context (Kozak, 1994, Biochimie 76:815-821; Jansen et al., 1994). 5' UTR with these characteristics should allow efficient 20 recognition of the 5' cap structure, followed by rapid and ribosome scanning by the ribosome, facilitating the translation initiation process.

The sequence of the synthetic 5'UTR was designed to be moderate in length (54 nucleotides (nts)), to be deficient in G but rich in C and A residues, to lack an upstream ATG, to place the intended ATG within the context of a optimal Kozak sequence (CCACCATGG), and to lack potential secondary structure. The synthetic 5' UTR sequence was also designed to lack AU-rich sequences that target mRNAs to be rapidly degraded in the cytoplasm.

Experiments have demonstrated that introns increase gene expression from cDNA vectors, and that introns located in the 5' UTR are more effective than ones located in the 3' UTR (Huang and Gorman, 1990, Mol. Cell. Biol. 10:1805-1810; Evans and Scarpulla, 1989, Gene 84:135-142; Brinster et al.,

1988, Proc. Natl. Acad. Sci. USA 85:836-840; Palmiter et al., 1991, Proc. Natl. Acad. Sci. USA 88:478-482; Choi et al., 1991, Mol. Cell. Biol. 11:3070-3074). Accordingly, the synthetic 5' UTR sequence was designed to accommodate an intron with consensus splice site sequences. The intron may, for example, be located between residues 48 and 49 (See intron sequence structure below). The CAG at position 46-48 is the exonic portion of a consensus 5' splice site. The G at position 49 is the exonic portion of a consensus 3' splice site.

To facilitate cloning manipulations, the synthetic 5' UTR sequence was designed to begin with a HindIII site and terminate with a NcoI site.

#### Features of the Synthetic Intron

RNA splicing is required for the expression of most eukaryotic genes. For optimal gene expression, RNA splicing must be highly efficient and accurate. A synthetic intron, termed OPTIVS8B, was designed to be maximally efficient and accurate.

The structure of the exemplary synthetic intron, OPTIVS8 is shown below. Sequences for the 5' splice site (5'ss), branch point (bp), and 3' splice site (3'ss) are double underlined. The recognition sequences for the restriction enzymes BbsI and EarI are overlined. The cleavage site for BbsI corresponds to the 5'ss, and the cleavage site for EarI corresponds to the 3'ss.

5'ss bp 3'ss | BbsI | EarI |

30 5'<u>CAG GTAAGT</u>GTCTTC---(77)---<u>TACTAAC</u>GG<u>TTCTTTTTTCTCTCACAG G</u> 3' (SEQ ID NO.15) (SEQ ID NO.16)

The 5' splice site (5'ss) sequence matches the established consensus sequence, MAG  $\square$  GTRAGT, where M = C or A, and R = G or A. Since the mechanism of splicing involves an interaction between the 5'ss of the pre-mRNA and U1

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snRNA, the 5'ss sequence of OPTIVS8B was chosen to be exactly complementary to the 5' end of U1 snRNA.

> 5'ss 5' CAGGUAAGU 3' 111111111 U1 RNA

In mammals, the consensus sequence for branch points (YNYTRAY, where Y = C or T, R = A or G, N = any base, andthe underlined A residue is the actual branch point) is very 10 Since the mechanism of splicing involves . ambiguous. interaction between the branch point (bp) of the pre-mRNA and U2 snRNA, the branch point sequence of OPTIVS8B was chosen to maximize this interaction. (Note that the branch point itself is bulged out). The chosen sequence also 15 matches the branch point sequence that is known to be obligatory for pre-mRNA splicing in yeast. The branch point is typically located 18-38 nts upstream of the 3' In OPTIVS8B, the branch point is located 24 nts 20 upstream from the 3' splice site.

3' GUCCAUUCA 5'

ΒP 5' UACUAAC 3' 11111 U2 RNA 3' AUGAU G 5'

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The sequence of the 3' splice site (3'ss) matches the established consensus sequence,  $Y_{11}NYAG \downarrow G$ , where Y = C or T, and N = any base. In 3' splice sites, the polypyrimidine tract  $(Y_{11})$  is the major determinant of splice site strength. For optimal splice site function in OPTIVS8B, the length of the polypyrimidine tract was extended to 16 bases, and its sequence was adjusted to contain 7 consecutive T residues. This feature was included because Roscigno et al. (1993) demonstrated that optimal splicing requires the presence of at least 5 consecutive T residues in the polypyrimidine tract.

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Splicing in vitro is generally optimal when introns are >80 nts in length (Wieringa, et al., 1984; Ulfendahl et al., 1985, Nucl. Acids Res. 13:6299-6315). Although many introns may be thousands of bases in length, most naturally occurring introns are 90-200 nt in length (Hawkins, 1988, Nucl. Acids Res. 16:9893-9908). The length of the synthetic intron (118 nts) falls within this latter range.

OPTIVS8B was designed with three internal restriction enzyme sites, BbsI, NheI, and EarI. These restriction sites facilitate the screening and identification of genes that contain the synthetic intron sequence. In addition, the BbsI and EarI sites were placed so that their cleavage sites exactly correspond to the 5'ss (BbsI) or 3'ss (EarI). sequence of the polypyrimidine tract was specifically designed to accommodate the EarI restriction Inclusion of the BbsI and EarI sites at these locations is useful because they permit the intron to be precisely deleted from a gene. They also permit the generation of an "intron cassette" that can be inserted at other locations within a gene.

The 77 bases between the BbsI site and the branch point sequence are random in sequence, except for the inclusion of the NheI restriction site.

## 4. Features of the Synthetic 3' UTR/poly(A) Signal:

The 3' ends of eukaryotic mRNAs are formed by the process of polyadenylation. This process involves site specific site RNA cleavage, followed by addition of a poly(A) tail. RNAs that lack a poly(A) tail are highly unstable. Thus, the efficiency of cleavage/polyadenylation is a major determinant of mRNA levels, and thereby, of gene expression levels. 2XPA1 is a synthetic sequence, containing two efficient poly(A) signals, that is designed to be maximally effective in polyadenylation.

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A poly(A) signal is required for the formation of the 3' end of most eukaryotic mRNA. The signal directs two RNA processing reactions: site-specific endonucleolytic cleavage of the RNA transcript, and stepwise addition of adenylates (approximately 250) to the newly generated 3' end to form the poly(A) A poly(A) signal has three parts: tail. hexanucleotide, cleavage site, and downstream element. hexanucleotide is typically AAUAAA and cleavage sites are most frequently 3' to the dinucleotide CA (Sheets et al., Downstream elements are required for optimal poly(A) 1987). signal function and are located downstream of the cleavage The sequence requirement for downstream elements is not yet fully established, but is generally viewed as UG- or sequences (Wickens, 1990; Proudfoot, 1991, 64:671-674; Wahle, 1992, Bioessays 14:113-118; Chen and Nordstrom, 1992, Nucl. Acids Res. 20:2565-2572).

Naturally occurring poly(A) signals are highly variable in their effectiveness (Peterson, 1992). The effectiveness of a particular poly(A) signal is mostly determined by the quality of the downstream element. (Wahle, 1992). In expression vectors designed to express genes of therapeutic interest, it is important to have a poly(A) signal that is as efficient as possible.

Poly(A) efficiency is important for gene expression, 25 because transcripts that fail to be cleaved polyadenylated are rapidly degraded in the compartment. In fact, the efficiency of polyadenylation in living cells is difficult to measure, nonpolyadenylated RNAs are so unstable. In addition to being required for mRNA stability, poly(A) tails contribute 30 to the translatability of mRNA, and may influence other RNA processing reactions such as splicing or RNA transport ((Jackson and Standart, 1990, Cell 62:15-24; Wahle, 1992).

Some eukaryotic genes have more than one poly(A) site, implying that if the cleavage/polyadenylation reaction fails to occur at the first site, it will occur at one of the

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later sites. In COS cell transfection experiments, a gene with two strong poly(A) sites yielded approximately two-fold more mRNA than one with a single strong poly(A) These data suggest that a significant (Bordonaro, 1995). fraction of transcripts remain unprocessed even with a single "efficient" poly(A) signal. Thus, it may be preferable to include more than one poly(A) site.

The sequence of the exemplary synthetic poly(A) signal below. The sequence is named 2XPA. shown hexanucleotide sequences and downstream element sequences are double underlined, and the two poly(A) sites are labeled Convenient restriction sites pA#1 and pA#2. The entire 2XPA unit may be transferred in overlined. cloning experiments as a XbaI-KpnI fragment. the internal BspHI fragment results in the formation of a 1XPA unit. (SEQ ID NO. 17)

<u>Bs</u>pHI XbaI

TCTAGAGCATTTTTCCCTCTGCCAAAAATTATGGGGACATCATGAAGCCCCTTGAGCATCTGACG pA#1

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Hex

1 TCTGGCT<u>AATAAA</u>GGAAATTTATTTTCATTGCAATA<u>GTGTGTTGGAATTTTTTGTGT</u>CTCTCACT

#### BspHI

Downstream element

25 CGGTACTAGAGCATTTTTCCCTCTGCCAAAAATTATGGGGACATCATGAAGCCCCTTGAGCATCT pA#2

Hex 1 Downstream element GACGTCTGGCT<u>AATAAA</u>GGAAATTTATTTTCATTGCAATA<u>GTGTGTTGGAATTTTTTGTGT</u>CTCT

30 KpnI CACTCGGTACC

The sequence of the synthetic poly(A) site shown above is based on the sequence of the rabbit □-globin poly(A) signal, a signal that has been characterized 35 literature as strong (Gil and Proudfoot, 1987, Cell 49:399-

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406; Gil and Proudfoot, 1984, Nature 312:473-474). One of its key features is the structure of its downstream element, which contains both UG- and U-rich domains.

A double-stranded DNA sequence corresponding to the 1XPA sequence was constructed from synthetic oligonucleotides. Two copies of the 1XPA sequence were then joined to form the 2XPA sequence. The sequences were joined in such as way as to have a unique XbaI site at the 5' end of the first poly(A) signal containing fragment, and a unique KpnI site at the 3' end of the second poly(A) signal containing fragment.

## C. Interferon Alpha and IL-12 Coding Sequences

The nucleotide sequence of a natural human interferon alpha coding sequences is known, and is provided below, along with a synthetic sequence which also codes for human interferon alpha. The same applies with respect to the IL-12 coding sequences.

In some cases, instead of the natural sequence coding for interferon alpha, it is advantageous to synthetic sequences which encode interferon alpha. 20 Such synthetic sequences have alternate codon usage from the sequence, and thus have dramatically nucleotide sequences from the natural sequence. particular, synthetic sequences can be used which have codon 25 usage at least partially optimized for expression in a The natural sequences do not have such optimal codon usage. Preferably, substantially all the codons optimized.

Optimal codon usage in humans is indicated by codon usage frequencies for highly expressed human genes, as shown 30 in Fig. The codon usage chart is from the program 3. "Human\_High.cod" from the Wisconsin Sequence Analysis Package, Version 8.1, Genetics Computer Group, Madison, WI. The codons which are most frequently used in highly expressed human genes are presumptively the optimal codons 35

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for expression in human host cells, and thus form the basis for constructing a synthetic coding sequence. An example of a synthetic interferon alpha coding sequence is shown as the bottom sequence in the table below.

However, rather than a sequence having fully optimized codon usage, it may be desirable to utilize an interferon alpha encoding sequence which has optimized codon usage except in areas where the same amino acid is too close together or abundant to make uniform codon usage optimal.

In addition, other synthetic sequences can be used which have substantial portions of the codon optimized, for example, with at least 50%, 70%, 80% or 90% optimized codons as compared to a natural coding sequence. Other particular synthetic sequences for interferon alpha can be selected by reference to the codon usage chart in Fig. 3. A sequence is selected by choosing a codon for each of the amino acids of the polypeptide sequences. molecules corresponding to each of the polypeptides can then by constructed by routine chemical synthesis methods. example, shorter oligonucleotides can be synthesized, and then ligated in the appropriate relationships to construct the full-length coding sequences.

The following sequences are provided in the sequence listing herein: interferon alpha amino acid sequence, SEQ ID NO:9; interferon alpha wild type nucleic acid sequence, ID NO:10; interferon alpha synthetic nucleic sequence with optimized codon usage, SEQ ΙD NO:11; interferon alpha nucleic acid sequence with additional/semioptimized codon usage, SEQ ID NO:12; IL-12 p40 subunit amino acid sequence, SEQ ID NO:1; IL-12 p40 wild type nucleic acid sequence, SEQ ID NO:2; IL-12 p40 synthetic nucleic acid sequence with all codons optimized, SEQ ID NO:3; IL-12 p40 subunit nucleic acid sequence with all codons optimized except when same nucleic acids were too close/abundant, SEQ ID NO:4; IL-12 p35 amino acid sequence, SEQ ID NO:5; IL-12 p35 wild type nucleic acid sequence, SEQ ID NO:6; IL-12 p35

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synthetic nucleic acid sequence with all codons optimized, SEQ ID NO:7; IL-12 p35 subunit nucleic acid sequence with all codons optimized except when same nucleic acids were too close/abundant, SEQ ID NO:8. Those skilled in the art will realize that various nucleic acid sequences with optimized codon usage can be constructed, for example based on the various combinations shown below, wherein optimal usage for each codon is shown below the IL-12 p35 and p40 subunit wild type sequences and the interferon alpha wild type sequence.

## 10 Sequences Encoding Human IL-12 p35

First line = natural sequence (SEQ ID=NO. 6)
Second line = all codons optimized (SEQ ID NO. 7)

Third line = all codons optimized except when same nucleic acids were too close/abundant (changes between second and third lines bolded) (SEQ ID NO. 8)

ATG TGT CCA GCG CGC AGC CTC CTC CTT GTG GCT ACC CTG GTC CTC CTG GAC CAC CTC ACT
ATG TGC CCC GCC CGC AGC CTG CTG CTG GTG GCC ACC CTG GTG CTG GAC CAC CTG AGC
ATG TGC CCC GCC CGC AGC CTG CTG CTG CTG GCC ACC CTG GTG CTC CTG GAC CAC CTC AGC

TTG GCC AGA AAC CTC CCC GTG GCC ACT CCA GAC CCA GGA ATG TTC CCA TGC CTT CAC CAC CTG GCC CGC AAC CTG CCC GTG GCC ACC CCA GAC CCC GGC ATG TTC CCA TGC CTG CAC CAC CTG GCC GCC AAC CTC CCC GTG GCC ACC CAC CCC GCC AAC CTC CCC GTG GCC CAC CAC

25 TCC CAA AAC CTG CTG AGG GCC GTC AGC AAC ATG CTC CAG AAG GCC AGA CAA ACT CTA GAA
AGC CAG AAC CTG CTG GCG GCC GTG AGC AAC ATG CTG CAG AAG GCC GCG CAG ACC CTG GAG
AGC CAG AAC CTG CTG GCG GCC GTG AGC AAC ATG CTG CAG AAG GCC GCG CAG ACC CTG GAG

TTT TAC CCT TGC ACT TCT GAA GAG ATT GAT CAT GAA GAT ATC ACA AAA GAT AAA ACC AGC TC TAC CCC TGC ACC AGC GAG GAG ATC GAC CAC GAG GAC ATC ACC AAG GAC AAG ACC AGC TC TAC CCC TGC ACC AGC GAG GAG ATC GAC CAC GAG GAC ATC ACC AAG GAC AAG ACC AGC

ACA GTG GAG GCC TGT TTA CCA TTG GAA TTA ACC AAG AAT GAG AGT TGC CTA AAT TCC AGA
ACC GTG GAG GCC TGC CTG CCC CTG GAG CTG ACC AAG AAC GAG AGC TGC CTG AAC AGC CGC

35 ACC GTG GAG GCC TGC CTG CCC CTC GAG TTA ACC AAG AAC GAG AGC TGC CTC AAC AGC CGC

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	GAG	ACC	TCT	TTC	ATA	ACT	AAT	GGG	AGT	TGC	CTG	GCC	TCC	AGA	AAG	ACC	TCT	TTT	ATG	ATG
	GAG	ACC	AGC	TTC	ATC	ACC	AAC	GGC	AGC	TGC	CTG	GCC	AGC	CGC	AAG	ACC	AGC	TTC	ATG	ATG
	GAG	ACC	TCC	TTC	ATC	ACC	AAC	GGC	ACT	TGC	CTG	GCC	TCC	CGC	AAG	ACC	AGC	TTC	ATG	ATG
5																				
	GCC	CTG	TGC	CTT	AGT	AGT	ATT	TAT	gaa	GAC	TTG	AAG	ATG	TAC	CAG	GTG	GAG	TTC	AAG	ACC
	GCC	CTG	TGC	CTG	AGC	AGC	ATC	TAC	GAG	GAC	CTG	AAG	ATG	TAC	CAG	GTG	GAG	TTC	AAG	ACC
	GCC	CTG	TGC	CTG	AGC	TCC	ATC	TAC	GAG	GAC	CTG	AAG	ATG	TAC	CAG	GTG	GAG	TTC	AAG	ACC
10	ATG	TAA	GCA	AAG	CTT	CTG	ATG	GAT	CCT	AAG	AGG	CAG	ATC	TTT	CTA	GAT	CAA	AAC	ATG	CTG
	ATG	AAC	GCC	AAG	CTG	CTG	ATG	GAC	ccc	AAG	CTC	CAG	ATC	TTC	CTG	GAC	CAG	AAC	ATG	CTG
	ATG	AAC	GCC	AAG	CTC	CTG	ATG	GAC	CCC	AAG	CTC	CAG	ATC	TTC	ÇTG	GAC	CAG	AAC	ATG	CTG
	GCA	GTT	ATT	GAT	GAG	CTG	ATG	CAG	GCC	CTG	AAT	TTC	AAC	AGT	GAG	ACT	GTG	CCA	CAA	AAA
15	GCC	GTG	ATC	GAC	GAG	CTG	ATG	CAG	GCC	CTG	AAC	TTC	AAC	AGC	GAG	ACC	GTG	CCC	CAG	AAG
	GCC	GTG	ATC	GAC	GAG	CTG	ATG	CAG	GCC	CTG	AAC	TTC	AAC	AGC	GAG	ACC	GTG	ccc	CAG	AAG
	TCC	TCC	CTT	GAA	GAA	CCG	GAT	TTT	TAT	AAA	ACT	AAA	ATC	AAG	CTC	TGC	ATA	CTT	CTT	CAT
	AGC	AGC	CTG	GAG	GAG	ccc	GAC	TTC	TAC	AAG	ACC	AAG	ATC	AAG	CTG	TGC	ATC	CTG	CTG	CAC
20	AGC	AGC	CTG	GAG	GAG	CCC	GAC	TTC	TAC	AAG	ACC	AAG	ATC	AAG	CTG	TGC	ATC	CTG	CTG	CAC
	GCT	TTC	AGA	TTA	CGG	GCA	GTG	ACT	ATT	GAC	AGA	GTG	ACG	AGC	TAT	CTG	AAT	GCT	TCC	TAA
	GCC	TTC	CGC	ATC	CGC	GCC	GTG	ACC	ATC	GAC	CGC	GTG	ACC	AGC	TAC	CTG	AAC	GCC	ACC	TGA
	GCC	TTC	CGC	ATC	CGG	GCC	GTG	ACC	ATC	GAC	CGC	GTG	ACC	AGC	TAC	CTG	AAC	GCC	ACG	TGA
25	Add	liti	ona	al C	)pti	.miz	ed	Sec	uer	ces	C C	dir	ig E	or	IL-	12	p35	Su	ıbur	<u>it</u>
	(Se	COL	nd I	ine	=	SEC	) II	NC	):24	1)										
										10										20
	Met	Суз	Pro	Ala	Arg	Ser	Leu	Leu	Leu	Val	Ala	Thr	Leu	Val	Leu	Leu	Asp	His	Leu	Ser
	ATG	TGY	CCN	GCN	MGN	WSN	YTN	YTN	YTN	GTN	GCN	ACN	YTN	GTN	YTN	YTN	GAY	CAY	YTN	WSN
30															<del>-</del>					
	ATG	TGT	CCT	GCT	CGT	TCT	TTA	TTA	TTA	GTT	GCT	ACT	TTA	GTT	TTA	TTA	GAT	CAT	TTA	TCT
		TGC	CCC	GCC	CGC	TCC	TTG	TTG	ТТG	GTC	GCC	ACC	TTG	GTC	TTG	TTG	GAC	CAC	TTG	TCC
			CCA	GCA	CGA	TCA	CTT	CTT	CTT	GTA	GCA	ACA	CTT	GTA	CTT	CTT			CTT	TCA
			000	000	000	maa	CMC	CMC	CTIC.	CEC	000	100	CMC	CmC	cmc	cmc.			-	

	AGA AGT CTA CTA	CTA CTA CTA CTA AGT
	AGG AGC CTG CTG	CTG CTG CTG AGC
	22	
5	Leu Ala Arg Asn Leu Pro Val Ala man Day	40
	Leu Ala Arg Asn Leu Pro Val Ala Thr Pro Asp YTN GCN MGN AAY YTN CCN GTN GCN ACN CCN GAY	p Pro Gly Met Phe Pro Cys Leu His His
		I CCN GGN ATG TTY CCN TGY YTN CAY CAY
	TTA GCT CGT AAT TTA CCT GTT GCT ACT CCT GAT	
	TTG GCC CGC AAC TTG CCC GTC GCC ACC CCC GAC	C CCC GGC TTG CGC TGT TTA CAT CAT
10	CTT GCA CGA CTT CCA GTA GCA ACA CCA	CCA CCA
	CTC GCG CGG CTC CCG GTG GCG ACG CCG	CCA GGA CCC CTT
	CTA AGA CTA	
	CTG AGG CTG	CTA CTG
		CIG
15	. 50	60
	Ser Gln Asn Leu Leu Arg Ala Val Ser Asn Met	Leu Gln Lys Ala Arg Gln Thr Lou Glu
	WIN CAR AAY YIN YTN MGN GCN GTN WSN AAY ATG	YTN CAR AAR GCN MGN CAR ACN YTN GAR
20	TCC CAC AND THE TA CGT GCT GTT TCT AAT ATG	TTA CAA AAA GCT CGT CAA ACT TTA GAA
20	THE CAG AAC TTG TTG CGC GCC GTC TCC AAC	TTG CAG AAG GCC CGC CAG ACC TTG GAG
	CTT CTT CGA GCA GTA TCA	CTT GCA CGA ACA CTT
	ACT COC CGG GCG GTG TCG	CTC GCG CGG ACG CTC
	OTA CTA AGA AGT	CTA AGA CTA
25		CTG AGG CTG
	Phe Tyr Pro Cys Thr Sor Clu Clu Ti	80
	Phe Tyr Pro Cys Thr Ser Glu Glu Ile Asp His C	Glu Asp Ile Thr Lys Asp Lys Thr Ser
	TTY TAY CCN TGY ACN WSN GAR GAR ATH GAY CAY C	GAR GAY ATH ACN AAR GAY AAR ACN WSN
30	TTT TAT CCT TGT ACT TCT GAA GAA ATT GAT CAT G	
	TTC TAC CCC TGC ACC TCC GAG GAG ATC GAC CAC G	GAA GAT ATT ACT AAA GAT AAA ACT TCT
	CCA ACA TCA ATA	
	CCG ACG TCG	ATA ACA ACA TCA
	AGT	ACG ACG TCG
35	AGC	AGT
		AGC

										90										100
	Thr	Val	Glu	Ala	Cys	Leu	Pro	Leu	Glu	Leu	Thr	Lys	Asn	Glu	Ser	Cys	Leu	Asn	Ser	Arg
	ACN	GTN	GAR	GCN	TGY	YTN	CCN	YTN	GAR	YTN	ACN	AAR	AAY	GAR	wsn	TGY	YTN	AAY	WSN	MGN
5	ACT	GTT	GAA	GCT	TGT	TTA	CCT	TTA	GAA	TTA	ACT	AAA	AAT	GAA	TCT	TGT	TTA	AAT	TCT	CGT
	ACC	GTC	GAG	GCC	TGC	TTG	ccc	TTG	GAG	TTG	ACC	AAG	AAC	GAG	TCC	TGC	TTG	AAC	TCC	CGC
	ACA	GTA		GCA		СТТ	CCA	СТТ		СТТ	ACA				TCA		CTT		TCA	CGA
	ACG	GTG		GCG		СТС	CCG	CTC		CTC	ACG				TCG		CTC		TCG	CGG
						СТА		CTA		CTA					AGT		CTA		AGT	AGA
10						CTG		CTG		CTG					AGC		CTG		AGC	AGG
										110					-					120
	Glu	Thr	Ser	Phe	Ile	Thr	Asn	Gly	Ser	Cys	Leu	Ala	Ser	Arg	Lys	Thr	Ser	Phe	Met	Met
	GAR	ACN	WSN	TTY	АТН	ACN	AAY	GGN	WSN	TGY	YTN	GCN	WSN	MGN	AAR	ACN	WSN	TTY	ATG	ATG
15																				
	GAA	ACT	TCT	TTT	ATT	ACT	AAT	GGT	тст	TGT	TTA	GCT	TCT	CGT	AAA	ACT	TCT	TTT	ATG	ATG
	GAG	ACC	TCC	TTC	ATC	ACC	AAC	GGC	TCC	TGC	TTG	GCC	TCC	CGC	AAG	ACC	TCC	TTC		
		ACA	TCA		ATA	ACA		GGA	TCA		CTT	GCA	TCA	CGA		ACA	TCA			
		ACG	TCG			ACG		GGG	TCG		CTC	GCG	TCG	CGG		ACG	TCG			
20			AGT						AGT		CTA		AGT	AGA			AGT			
			AGC						AGC		CTG		AGC	AGG			AGC			
										130										140
	Ala	Leu	Cys	Leu	Ser	Ser	Ile	Tyr	Glu	Asp	Leu	Lys	Met	Tyr	Gln	Val	Glu	Phe	Lys	Thr
25	GCN	YTN	TGY	YTN	WSN	WSN	ATH	TAY	GAR	GAY	YTN	AAR	ATG	TAY	CAR	GTN	GAR	TTY	AAR	ACN
	GCT	TTA	TGT	TTA	TCT	TCT	ATT	TAT	GAA	GAT	TTA	AAA	ATG	TAT	CAA	GTT	GAA	TTT	AAA	ACT
	GCC	TTG	TGC	TTG	TCC	TCC	ATC	TAC	GAG	GAC	TTG	AAG		TAC	CAG	GTC	GAG	TTC	AAG	ACC
	GCA	CTT		CTT	TCA	TCA	ATA				CTT					GTA				ACA
30	GCG	CTC		СТС	TCG	TCG					СТС					GTG				ACG
		CTA		СТА	AGT	AGT					СТА									
		CTG		CTG	AGC	AGC					CTG									

										15	50										
	Met	Asn	Ala	Lys	Leu	Leu	Met	Asp	Pr			Ara (	G I n	710	DL.	•	_				160 Leu
	ATG	AAY	GCN	AAR	YTN	YTN	ATG	GAY	CC	AA N	AR M	ign (	מב	מידת	THE	ren	Asp	Gln	Asn	Met	Leu YTN
									<b>-</b>					A111		ITN	GAY	CAR	AAY	ATO	YTN
5	ATG	AAT	GCT	AAA	TTA	TTA	ATG							7 mm i							TTA
		AAC	GCC	AAG	TTG	TTG		GAC	cco	: AA	G C	GC (	יאה י	MTC (	TTT	TTG (	GAT	CAA	ААТ	ATG	TTA
			GCA		CTT	CTT			CCF			GA		ATA			GAC	CAG	AAC		TTG
			GCG		СТС	CTC						GG		41A		CTT					CTT
					СТА	СТА					A					CTC					CTC
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	GCC G	TC A	TC G	AC G	AG 1	TG	c	AG (	GCC	TTG	ממ		. A4	AT TO	T G	AA A	CT G	TT C	CT C	AA .	AAA
	GCA G	TA A	TA		c	TT			GCA	СТТ		- 11	CAA							AG A	AAG
	GCG G					TC			GCG					TC		AC					
20					¢	TA				CTA				TC		AC	G G1	rg co	CG		
-					С	TG				CTG				AG							
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	Ser Se WSN WS	r Le	eu Gi	lu G]	iu Pi	ro As	p Pi	ne T			Th ►	Tue	т1.		_					2	00
25	WSN WS	N YI	'N GA	IR GA	IR CO	ON GA	- Y T7	Y T	3 Ay 2	ΔR	ארא	מאא	116	· Ly:	s Le	u Cy:	s Il	e Le	u Le	u H	is
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	TCT TC																- <del>-</del> -				
	TCA TCA	с тт	G GA	G GA	G CC	C GA	СТТ	C Tz	AC A	AG Z	*CC	ממא	ATT	AAA	TT	A TGT	'AT'	r TT	A TT	A C	AT
	TCA TC	A CT	Т		CC	A					ACA										AC.
30	TCG TCC	CT	С		CC	G					ACG		ATA	•			ATA	A CTT	CT	r	
	AGT AGT	СТ	Ą							-	.00				CTC			CTO	CT	3	
	AGC AGC	CTO	3												CTA			CTA	CTA	A	
															CTG			CTG	CTC	;	

										210										220
	Ala	Phe	Arg	Ile	Arg	Ala	Val	Thr	Ile	Asp	Arg	Val	Thr	Ser	Tyr	Leu	Asn	Ala	Ser	***
	GCN	TTY	MGN	ATH	MGN	GCN	GTN	ACN	HTA	GAY	MGN	GTN	ACN	WSN	TAY	YTN	AAY	GCN	WSN	TRR
																				- <b>-</b> -
5	GCT	TTT	CGT	ATT	CGT	GCT	GTT	ACT	ATT	GAT	CGT	GTT	ACT	TCT	TAT	TTA	AAT	GCT	TCT	TAA
	GCC	TTC	CGC	ATC	CGC	GCC	GTC	ACC	ATC	GAC	CGC	GTC	ACC	TCC	TAC	TTG	AAC	GCC	TCC	TAG
	GÇA		CGA	ATA	CGA	GCA	GTA	ACA	ATA		CGA	GTA	ACA	TCA		CTT		GCA	TCA	TGA
	GCG		CGG		CGG	GCG	GTG	ACG			CGG	GTG	ACG	TCG		CTC		GCG	TCG	
			AGA		AGA						AGA			AGT		СТА			AGT	
10			AGG		AGG						AGG			AGC		CTG			AGC	
	Sec	juer	ices	s Er	100	ding	g Hu	ımar	ı II	L-12	2 p4	10			2					
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15	nuc	:lei	ic	aci	ids	W	ere	t	00	cl	ose	/ab	und	ant	(	cha	nge	s	bet	ween
	sec	conc	i ar	nd t	hii	d I	line	es k	oolo	ded)	( 5	SEQ	ID	NO.	. 4)					
	ATG	TGT	CAC	CAG	CAG	TTG	GTC	ATC	TCT	TGG	TTT	TCC	CTG	GTT	TTT	CTG	GCA	TCT	ccc	ÇTC
	ATG	TGC	CAC	CAG	CAG	CTG	GTG	ATC	AGC	TGG	TTC	AGC	CTG	GTG	TTC	CTG	GCC	AGC	CCC	CTG
	ATG	TGC	CAC	CAG	CAG	CTG	GTG	ATC	AGC	TGG	TTC	TCC	CTG	GTG	TTT	CTG	GCC	AGC	CCC	CTC
20																				
	GTG	GCC	ATA	TGG	GAA	CTG	AAG	AAA	GAT	GTT	TAT	GTC	GTA	GAA	ТŢG	GAT	TGG	TAT	CCG	GAT
	GTG	GCC	ATC	TGG	GAG	CTG	AAG	AAG	GAC	GTG	TAC	GTG	GTG	GAG	CTG	GAC	TGG	TAC	ccc	GAC
	GTG	GCC	ATC	TGG	GAG	CTG	AAG	AAA	GAC	GTG	TAC	GTG	GTC	GAG	CTG	GAC	TGG	TAC	ccc	GAC
25	GCC	CCT	GGA	GAA	ATG	GTG	GTC	CTC	ACC	TGT	GAC	ACC	CCT	GAA	GAA	GAT	GGT	ATC	ACC	TGG
	GCC	ccc	GGC	GAG	ATG	GTG	GTG	CTG	ACC	TGC	GAC	ACC	ccc	GAG	GAG	GAC	GGC	ATC	ACC	TGG
	GCC	CCC	GGC	GAG	ATG	GTG	GTC	CTG	ACC	TGC	GAC	ACC	ccc	GAG	GAA	GAC	GGC	ATC	ACC	TGG
	ACC	<b>T</b> TG	GAC	CAG	AGC	AGT	GAG	GTC	TTA	GGC	TCT	GGC	AAA	ACC	CTG	ACC	ATC	CAA	GTC	AAA
30	ACC	CTG	GAC	CAG	AGC	AGC	GAG	GTG	CTG	GGC	AGC	GGC	AAG	ACC	CTG	ACC	ATC	CAG	GTG	AAG
	ACC	CTG	GAC	CAG	AGC	AGT	GAG	GTG	CTG	GGC	TCC	GGC	AAG	ACC	CTG	ACC	ATC	CAG	GTG	AAG
	GAG	TTT	GGA	GAT	GCT	GGC	CAG	TAC	ACC	TGT	CAC	AAA	GGA	GGC	GAG	GTT	CTA	AGC	CAT	TCG
	GAG	TTC	GGC	GAC	GCC	GGÇ	CAG	TAC	ACC	TGC	CAC	AAG	GGC	GGC	GAG	GTG	CTG	AGC	CAC	AGC
35	GAG	TTC	GGC	GAC	GCC	GGC	CAG	TAC	ACC	TGC	CAC	AAG	GGA	GGC	GAG	GTG	CTG	AGC	CAC	TCC

CTC CTG CTG CTG CAC AAA AAG GAA GAT GGA ATT TGG TCC ACT GAT ATT TTA AAG GAC CAG
CTG CTG CTG CTG CAC AAG AAG GAG GAC GGC ATC TGG AGC ACC GAC ATC CTG AAG GAC CAG
CTC CTG CTG CTG CAC AAA AAG GAG GAC GGC ATC TGG AGC ACC GAC ATC CTG AAG GAC CAG

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AAA GAA CCC AAA AAT AAG ACC TTT CTA AGA TGC GAG GCC AAG AAT TAT TCT GGA CGT TTC
AAG GAG CCC AAG AAC AAG ACC TTC CTG CGC TGC GAG GCC AAG AAC TAC AGC GGC CGC TTC
AAG GAG CCC AAG AAC AAG ACC TTC CTG CGC TGC GAG GCC AAG AAC TAC AGC GGC CGC TTC

- ACC TGC TGG TGG CTG ACG ACA ATC AGT ACT GAT TTG ACA TTC AGT GTC AAA AGC AGC AGA
  ACC TGC TGG TGG CTG ACC ACC ATC AGC ACC GAC CTG ACC TTC AGC GTG AAG AGC AGC AGG
  ACC TGC TGG TGG CTG ACC ACG ATC AGC ACC GAC CTG ACC TTC AGT GTG AAG AGC AGC AGG
- GGC TCT TCT GAC CCC CAA GGG GTG ACG TGC GGA GCT GCT ACA CTC TCT GCA GAG AGA GTC

  15 GGC AGC AGC GAC CCC CAG GGC GTG ACC TGC GGC GCC GCC ACC CTG AGC GCC GAG CGC GTG

  GGC TCC AGC GAC CCC CAG GGC GTG ACC TGC GGC GCT GCC ACC CTG AGC GCC GAG CGC GTG

AGA GGG GAC AAC AAG GAG TAT GAG TAC TCA GTG GAG TGC CAG GAG GAC AGT GCC TGC CCA CGC GGC GAC AAC AAG GAG TAC GAG TAC AGC GTG GAG TGC CAG GAG GAC AGC GCC TGC CCC 20 CGC GGC GAC AAC AAG GAG TAC GAG TAC AGC GTG GAG TGC CAG GAA GAC TCC GCC TGC CCC

GCT GAG GAG AGT CTG CCC ATT GAG GTC ATG GTG GAT GCC GTT CAC AAG CTC AAG TAT
GCC GCC GAG GAG AGC CTG CCC ATC GAG GTG ATG GTG GAC GCC GTT CAC AAG CTG AAG TAC
GCC GCT GAG GAG AGC CTG CCC ATC GAG GTG ATG GTG GAC GCC GTT CAC AAG CTG AAG TAC

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GAA AAC TAC ACC AGC AGC TTC TTC ATC AGG GAC ATC ATC AAA CCT GAC CCA CCC AAG AAC GAG AAC TAC ACC AGC AGC TTC TTC ATC CGC GAC ATC ATC AAG CCC GAC CCC AAG AAC GAG AAC TAC ACC AGC AGC AGC TTC TTC ATC CGC GAC ATC ATC AAG CCT GAC CCA AGG AAC

THE CAG CTG AAG CCA TTA AAG AAT TCT CGG CAG GTG GAG GTC AGC TGG GAG TAC CCT GAC
CTG CAG CTG AAG CCC CTG AAG AAC AGC CGC CAG GTG GAG GTG AGC TGG GAG TAC CCC GAC
CTC CAG CTG AAG CCC CTC AAG AAC TCC CGC CAG GTG GAG GTG AGC TGG GAG TAC CCC GAC
ACC TGG AGT ACT CCA CAT TCC TAC TTC TCC CTG ACA TTC TGC GTT CAG GTC CAG GGC AAG
ACC TGG AGC ACC CCC CAC AGC TAC TTC TCC CTG ACC TTC TGC GTG CAG GTG CAG GGC AAG
35 ACC TGG AGC ACG CCC CAC TCC TAC TTC TCC CTG ACC TTC TGC GTG CAG GTC CAG GGC AAG

AGC AAG AGA GAA AAG AAA GAT AGA GTC TTC ACG GAC AAG ACC TCA GCC ACG GTC ATC TGC AGC AAG CGC GAG AAG AAG GAC CGC GTG TTC ACC GAC AAG ACC AGC GCC ACC GTG ATC TGC AGC AAG CGC GAG AAG AAA GAC CGG GTG TTC ACC GAC AAG ACC AGC GCC ACC GTC ATC TGC

CGC AAA AAT GCC AGC ATT AGC GTG CGG GCC CAG GAC CGC TAC TAT AGC TCA TCT TGG AGC CGC AAG AAC GCC AGC ATC AGC GTG CGC GCC CAG GAC CGC TAC TAC AGC AGC AGC TGG AGC CGC AAG AAC GCC AGC ATC AGC GTG CGC GCC CAG GAC CGC TAC TAT AGC TCC TCT TGG AGC

GAA TGG GCA TCT GTG CCC TGC AGT TAG GAG TGG GCC AGC GTG CCC TGC AGC TAG GAG TGG GCC AGC GTG CCC TGC TCC TAG

> CTA CTG

## Additional Optimized Sequences Coding For IL-12 p40 Subunit

(Second Line = SEQ ID NO:25) 20 Met Cys His Gln Gln Leu Val Ile Ser Trp Phe Ser Leu Val Phe Leu Ala Ser Pro Leu 15 ATG TGY CAY CAR CAR YTN GTN ATH WSN TGG TTY WSN YTN GTN TTY YTN GCN WSN CCN YTN \_\_\_ \_\_ \_\_ \_\_ \_\_ \_\_ \_\_ \_\_ \_\_ \_\_ \_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ ATG TGT CAT CAA CAA TTA GTT ATT TCT TGG TTT TCT TTA GTT TTT TTA GCT TCT CCT TTA 20 CTT GCA TCA CCA CTT TCA CTT GTA CTT GTA ATA TCA TCG CTC GTG CTC GCG TCG CCG CTC CTC GTG TCG CTA AGT AGT CTA CTA AGT AGC CTG CTG AGC CTG CTG AGC 25 30 Val Ala Ile Trp Glu Leu Lys Lys Asp Val Tyr Val Val Glu Leu Asp Trp Tyr Pro Asp GTN GCN ATH TGG GAR YTN AAR AAR GAY GTN TAY GTN GTN GAR YTN GAY TGG TAY CCN GAY --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---GAG TTG AAG AAG GAC GTC TAC GTC GTC GAG TTG GAC TAC CCC GAC 30 GTC GCC ATC GTA GCA ATA CTT GTA GTA GTA CTT CCA GTG GCG GTG GTG GTG CTC CCG CTC

CTA

CTG

35

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	Ala Pro G	ly Glu M	et Val	Val	Leu 1	hr C	ys A:	sp Th	r Pr	o (G)	u 61		- 01			60
	GCN CCN G	GN GAR A	TG GTN	GTN	YTN A	CN TO	- GY G	ייי י	N CC		D 61	u As	ь сту	Ile	Thr	Trp
					<b>-</b>		<b>-</b> .	·	.,	N GA	K GA	R GA	Y GGN	ATH	ACN	TGG
5	GCT CCT GG	GT GAA A	rg gtt	GTT 1	מ מיד	ርጥ ጥረ	מיי כיו		<b>-</b> .							
	GCC CCC GC	GC GAG	GTC	GTC 1	מ פרים	CC TC	31 G#	T AC	r cci	r GA	A GA	A GA	GGT	ATT	ACT	TGG
	GCC CCC GC GCA CCA GC	GA	GTA	GTA C	~~~ ~	CC 16					G GA	G GAC	GGC	ATC	ACC	
	GCG CCG GG							ACA					GGA	ATA	ACA	
			GTG			CG		ACC	CCG				GGG		ACG	
10					TA											
				С	TG											
	The tau a					70					*					80
	Thr Leu As	p Gln Se:	r Ser (	Glu V	al Le	u Gly	y Sei	Gly	Lys	Thr	Leu	Thr	Ile	Gln '	Val	I.ve
15		- CAR WSI	N WSN (	JAR G	IN YT	N GGN	WSN	GGN	AAR	ACN	VTM	N CNI	3 m.	CAR (	STN 2	0 A D
13												_				
	ACT TTA GAT	CAA TCT	TCT	SAA GI	T TT	A GGT	' TCT	GGT	AAA	ውግል	ጥጥአ	) Cm	<b>.</b>	ממי		
	ACC TTG GAC	CAG TCC	TCC G	AG GT	C TTO	GGC	TCC	GGC	AAG	ACC	TTG	ACC	( атс с	-AA. (	.mo -	LAA
	ACA CTT	TCA	TCA	GT	А СТІ	GGA	TCA	GGA		ACA	ייייט	200	A10 (	AG G		LAG
	ACG CTC	TCG	TCG	GT	G CTC	GGG	TCG	GGG		ACC	CTC	ACC.	MIM			
20	CTA	AGT	AGT		CTA		AGT		•			ACG		G	TG	
	CTG	AGC	AGC		CTG						CTA					
											CTG					
						90										
	Glu Phe Gly	Asp Ala	Glv Gl	ln Tv	- ምክ፦		175 -	<b>.</b> .							10	00
25	Glu Phe Gly GAR TTY GGN	GAY GCN	GGN C	יאידי ס		Cys	nis	Lys (	sly G	ly (	Glu '	Val I	eu S	er Hi	s Se	er
	GAR TTY GGN			u ini	ACN	TGY	CAY	AAR (	GN G	GN (	SAR (	SŢN Y	TN W	SN CA	Y WS	SN
	GAA TTT GGT	ርልጥ ርርጥ										<b>-</b> -				· <b>-</b>
	GAA TTT GGT	Che cee	GGT CA	A TAT	ACT	TGT	CAT	AAA G	GT G	GT G	AA (	тт т	TA TO	T CA	т тс	T
		GAC GCC	GGC CA	G TAC	ACC	TGC (	CAC	AAG G	GC G	GC G	AG G	тс т	TG TC	C CA	СТС	c
30	50.1	GCA	GGA		ACA				GA G				TT TC		TC	
	GGG	GCG (	GGG		ACG			G	GG G	GG .					TC	
													ra ag			
													rg ag			
													G AG	_	AG	L

										110										120
	Leu	Leu	Leu	Leu	His	Lys	Lys	Glu	Asp	Gly	Ile	Trp	Ser	Thr	Asp	Ile	Leu	Lys	Asp	Gln
	YTN	YTN	YTN	YTN	CAY	AAR	AAR	GAR	GAY	GGN	ATH	TGG	WSN	ACN	GAY	АТН	YTN	AAR	GAY	CAR
						<u></u>				· 										
5	TTA	ATT	TTA	TTA	ĊAT	AAA	AAA	GAA	GAT	GGT	ATT	TGG	TCT	ACT	GAT	ATT	TTA	AAA	GAT	CAA
	TTG	TTG	TTG	TTG	CAC	AAG	AAG	GAG	GAC	GGC	ATC		TCC	ACC	GAC	ATC	TTG	AAG	GAC	CAG
	CTT	CTT	CTT	CTT						GGA	ATA		TCA	ACA		ATA	СТТ			
	CTC	CTC	CTC	CTC						GGG			TCG	ACG			СТС			
	CTA	СТА	CTA	CTA									AGT				СТА			
10	CTG	CTG	CTG	CTG									AGC				CTG			
										130					2					140
	Lys	Glu	Pro	Lys	Asn	Lys	Thr	Phe	Leu	Arg	Cys	Glu	Ala	Lys	Asn	Tyr	Ser	Gly	Arg	Phe
	AAR	GAR	CCN	AAR	AAY	AAR	ACN	TTY	YTN	MGN	TGY	GAR	GCN	AAR	AAY	TAY	WSN	GGN	MGN	TTY
15																				
	AAA	GAA	CCT	AAA	AAT	AAA	ACT	TTT	TTA	CGT	TGT	GAA	GCT	AAA	AAT	TAT	TCT	GGT	CGT	TTT
	AAG	GAG	CCC	AAG	AAC	AAG	ACC	TTC	TTG	CGC	TGC	GAG	GCC	AAG	AAC	TAC	TCC	GGC	CGC	TTC
			CCA				ACA		CTT	CGA			GCA				TCA	GGA	CGA	
			CÇG				ACG		CTC	CGG			GCG				TÇG	GGG	CGG	
20									CTA	AGA							AGT		AGA	
									CTG	AGG							AGC		AGG	
										150										160
															Ser					
25	ACN	TGY	TGG	TGG	YTN	ACN	ACN	HTA	WSN	ACN	GAY	YTN	ACN	TTY	WSN	GTN	AAR	WSN	WSN	MGN
	ACT	TGT	TGG	TGG											TCT					
	ACC	TGC			TTG	ACC	ACC	ATC	TCC	ACC	GAC	TTG	ACC	TTC	TCC	GTC	AAG	TCC	TCC	CGC
	ACA				CTT	ACA	ACA	ATA	TCA	ACA		CTT	ACA		TCA	GTA		TCA	TCA	CGA
30	ACG				CTC	ACG	ACG		TCG	ACG		CTC	ACG		TCG	GTG		TCG	TCG	CGG
					CTA				AGT			CTA			AGT			AGT	AGT	AGA
					CTG				AGC			CTG			AGC			AGÇ	AGC	AGG

	170
	Gly Ser Ser Asp Pro Gln Gly Val Thr Cys Gly Ala Ala Thr Leu Ser Ala Glu Arg Val
	GGN WSN WSN GAY CCN CAR GGN GTN ACN TGY GGN GCN GCN ACN YTN WSN GCN GAR MGN GTN
	THE TOT GGN GCN ACN YTN WSN GCN GAR MGN GTN
5	GGT TCT TCT GAT CCT CAA GGT GTT ACT TGT GGT GCT GCT ACT TTA TCT GCT GAA CGT GTT
	GGC TCC TCC GAC CCC CAG GGC GTC ACC TGC GGC GCC ACC TTG TCC GCC GAG CGC GTC
	GGA TCA TCA CCA GGA GTA ACA GGA GCA GCA ACA CTT TCA GCA CGA GTA
	GGG TCG TCG CCG GGG GTG ACG GGG GCG GCG ACG CTC TCG GCG CGG GTG  AGT ACT
	1101
10	AGC AGC
	CTG AGC AGG
	190 -
	Arg Gly Asp Asn Lys Glu Tyr Glu Tyr Ser Val Glu Cys Gln Glu Asp Ser Ala Cys Pro
	THE TAR GAR TAY GAR TAY WSN GTN GAR TGY CAR CAR CAR
15	
	CGI GGT GAT AAT AAA GAA TAT GAA TAT TCT GTT GAA TGT CAA CAA CAA CAA
	CGC GGC GAC AAC AAG GAG TAC GAG TAC TCC GTC GAG TGC CAG GAG GAC TCC GCC TGC CCC
	TCA GTA
0.0	TCG GTG
20	AGA AGT AGT
	AGC AGC
•	210
25	Ala Glu Glu Ser Leu Pro Ile Glu Val Met Val Asp Ala Val Vic V
23	SAR WAS ITN CCN ATH GAR GTN ATG GTN GAY GCN GTN CAY ARD WITH THE
	GCC GCC GAG GAC TCC TTA CCT ATT GAA GTT ATG GTT GAT GCT GTT CAT AAA TTA AAA TAT
	GTC GAC GCC GTC CAC AAG TTG AAG TAC
	TCA CTT CCA ATA GTA GTA GCA GTA CTT
50	GCG GCG TCG CTC CCG GTG GTG GCG GTG CTC
	AGT CTA
	AGC CTG

										230										240
	Glu i	Asn	Tvr	Thr	Ser	Ser	Phe	Phe	Ile	Arg	Asp	Ile	Ile	Lys	Pro	Asp	Pro	Pro	Lys	Asn
	GAR																			
				<b>-</b>														<del>-</del>		
·5	GAA .	AAT	TAT	ACT	TCT	TCT	TTT	TTT	ATT	CGT	GAT	TTA	ATT	AAA	CCT	GAT	CCT	CCT	AAA	TAA
	GAG .																			
				ACA	TCA	TCA			ATA	CGA		ATA	ATA		CCA		CCA	CCA		
				ACG	TCG	TCG				CGG					CCG		CCG	CCG		
					AGT	AGT				AGA										
10					AGC	AGC				AGG										
										250					-					260
	Leu	Gln	Leu	Lys	Pro	Leu	Lys	Asn	Ser	Arg	Gln	Val	Glu	Val	Ser	Trp	Glu	Tyr	Pro	Asp
	YTN	CAR	YTN	AAR					WSN											
15																				
									TCT											
	TTG	CAG	TTG	AAG	ccc	TTG	AAG	AAC	TCC	CGC	CAG	GTC	GAG	GTC	TCC		GAG	TAC	ccc	GAC
	CTT		CTT		CCA	стт			TCA	CGA		GTA		GTA	TCA				CCA	
	CTC		CTC	:	CCG	CTC			TCG	CGG		GTG		GTG	TCG				CCG	
20	CTA		CTA	١		CTA	ı		AGT	AGA					AGT					
	CTG		CTG	;		CTG	i		AGC	AGG	i				AGC	•				
										270										280
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25														TGY	' GTN	CAF	GTN	CAF	GGN	AAR I
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	ACT	TGC																		AAA 1
	ACC	;	TC	CAC	c cc	CAC	TCC	TAC	C TTC	TCC	TTC	ACC								AAG
	ACA		TC	A AC	A CCI	A	TC	A		TC	A CT	r ACA					GT		GG/	
30	ACG	;	TC	G AC	G CC							CAC	3		GTO	3	GT	3	GG	ف
			AG	T			AG	r		AG.	r CT	A								
			AG	С			AG	2		AG	CT	G								

5	TCT AAA CGT GAA	AAA AAA GAT CGT ( AAG AAG GAC CGC C	GTG ACG ACG TCG GCG ACG GTG
10	AGC AGG	AGG	AGT AGC
15 20	CGT AAA AAT GCT TO CGC AAG AAC GCC TO CGA GCA TO CGG GCG TO AGA AG	CT ATT TCT GTT CG CC ATC TCC GTC CG CA ATA TCA GTA CG CC TCG GTG CGC	310  Arg Ala Gln Asp Arg Tyr Tyr Ser Ser Ser Trp Ser  IGN GCN CAR GAY MGN TAY TAY WSN WSN WSN TGG WSN   GT GCT CAA GAT CGT TAT TAT TCT TCT TCT TGG TCT  GC GCC CAG GAC CGC TAC TAC TCC TCC TCC  GA GCA CGA TCA TCA TCA TCA  GG GCG CGG TCG TCG  AGA AGA AGT AGT AGT AGT
25 .	SAA TGG GCT TCT GTT	N CCN TGY WSN TRR CCT TGT TCT TAA CCCC TGC TCC TAG CCCA TCA TGA	R - A G

63 72 81 90 99 108  AGC TGC TCT GTG GGC TGT GAT CTG CCT CAA ACC CAC AGC CTG GGT AGC AGG AGC  10 S C S V G C D L P Q T H S L G S R  117 126 135 144 153 162  ACC TTG ATG CTC CTG GCA CAG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGC AGG AGA ATC TCT CTT TTC TCC TGC TTG AGC AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT TTC TCC TGC TTG AGG AGA ATC TCT TTC TCT TTC TCC TGC TTG AGG AGA ATC TCT TTC TCC TGC TTG AGG AGA ATC TCT TTC TCT TTC TCC TGC TTG AGG AGA ATC TCT TTC TCC TGC TGC AGG AGA ATC TCT TTC TCT TTC TCC TGC TGC AGG AGA ATC TCT TTC TCC TGC TGC AGG AGA ATC TCT TTC TCC TGC TGC AGG AGC AGC AGG AGC AGC AGG AGC AGC A	·Cr
5 M A L T F A L L V A L L V L S C K  63 72 81 90 99 108  AGC TGC TCT GTG GGC TGT GAT CTG CCT CAA ACC CAC AGC CTG GGT AGC AGG AGC AGC AGC AGC AGC AGC AGC AGC	
63 72 81 90 99 108  AGC TGC TCT GTG GGC TGT GAT CTG CCT CAA ACC CAC AGC CTG GGT AGC AGG AGC  10 S C S V G C D L P Q T H S L G S R  117 126 135 144 153 162  ACC TTG ATG CTC CTG GCA CAG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGC AGG AGA ATC TCT CTT TTC TCC TGC TTG AGC AGG AGA ATC TCT CTT TTC TCC TGC TTG AGC AGG AGA ATC TCT CTT TTC TCC TGC TTG AGC AGG AGA ATC TCT CTT TTC TCC TGC TTG AGC AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT TTC TCC TGC TTG AGG AGA ATC TCT TTC TCC TGC TTG AGG AGA ATC TCT TTC TCC TGC TGC TGC AGG AGA ATC TCT TTC TCC TGC TGC TGC AGG AGA ATC TCT TCT TTC TCC TGC TGC AGG AGA ATC TCT TTC TCC TGC TGC AGG AGA ATC TCT TTC TCC TGC TGC AGG AGG AGC AGG AGC AGG AGC AGG AGC AGG AGG	CA
63 72 81 90 99 108  AGC TGC TCT GTG GGC TGT GAT CTG CCT CAA ACC CAC AGC CTG GGT AGC AGG AGC  10 S C S V G C D L P Q T H S L G S R  117 126 135 144 153 162  ACC TTG ATG CTC CTG GCA CAG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGC AGG AGA ATC TCT CTT TTC TCC TGC TTG AGC AGG AGA ATC TCT CTT TTC TCC TGC TTG AGC AGG AGA ATC TCT CTT TTC TCC TGC TTG AGC AGG AGA ATC TCT CTT TTC TCC TGC TTG AGC AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT TTC TCC TGC TTG AGG AGA ATC TCT TTC TCC TGC TTG AGG AGA ATC TCT TTC TCC TGC TGC TGC AGG AGA ATC TCT TTC TCC TGC TGC TGC AGG AGA ATC TCT TCT TTC TCC TGC TGC AGG AGA ATC TCT TTC TCC TGC TGC AGG AGA ATC TCT TTC TCC TGC TGC AGG AGG AGC AGG AGC AGG AGC AGG AGC AGG AGG	
AGC TGC TCT GTG GGC TGT GAT CTG CCT CAA ACC CAC AGC CTG GGT AGC AGG AGG AGG AGG AGC AGG AGG AGG AGG	s
AGC TGC TCT GTG GGC TGT GAT CTG CCT CAA ACC CAC AGC CTG GGT AGC AGG AGG AGG AGG AGC AGG AGG AGG AGG	
10 S C S V G C D L P Q T H S L G S R  117 126 135 144 - 153 162  ACC TTG ATG CTC CTG GCA CAG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG CTC TTG ATG CTC TTG ATG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG ATG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT TTC TCT TTC TCC TTG AGG AGG AGA ATC TCT TCT TTC TCC TGC TTG AGG AGG AGA ATC TCT TCT TTC TCC TGC TTG AGG AGG AGA ATC TCT TCT TCT TTC TCC TGC TTG AGG AGG AGG AGG AGG AGG AGG AGG AGG	
10 S C S V G C D L P Q T H S L G S R  117 126 135 144 - 153 162  ACC TTG ATG CTC CTG GCA CAG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG T L M L L A Q M R R I S L F S C L  171 180 189 198 207 216	iGG
117 126 135 144 - 153 162  ACC TTG ATG CTC CTG GCA CAG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG  T L M L L A Q M R R I S L F S C L .	R
ACC TTG ATG CTC CTG GCA CAG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT CTT TTC TCC TGC TTG AGG AGA ATC TCT TTC TCT TTC TCC TGC TTG AGG AGA ATC TCT TTC TCT TTC TTC TTC TTC TTC TT	K
15 T L M L L A Q M R R I S L F S C L .	?
171 180 189 198 207 216	<b>\A</b> G
171 180 189 198 207 216	
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GAC AGA CAT GAC TTT GGA TTT CCC CAG GAG GAG TTT GGC AAC CAG TTC CAA A	<b>L</b> AG
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20 D R H D F G F P Q E E F G N Q F Q	ĸ
225 234 243 252 261 270	)
GCT GAA ACC ATC CCT GTC CTC CAT GAG ATG ATC CAG CAG ATC TTC AAT CTC T	TC
25 AETIPVLHEMIQQIFNL	F
, 279 288 297 306 315 324	1
AGC ACA AAG GAC TCA TCT GCT GCT TGG GAT GAG ACC CTC CTA GAC AAA TTC T	rac
30 STKDSSAAWDETLLDKF	Y
333 342 351 360 369 378	
ACT GAA CTC TAC CAG CAG CTG AAT GAC CTG GAA GCC TGT GTG ATA CAG GGG (	
35 TELYQQLNDLEACVIQG	

GGG GTG ACA GAG ACT CCC CTG ATG AAG GAG GAC TCC ATT CTG GCT GTG AGG AAA --- --- --- --- --- --- --- --- --- --- --- --- ---G V T E T P L M K E D S I L A V R TAC TTC CAA AGA ATC ACT CTC TAT CTG AAA GAG AAG AAA TAC AGC CCT TGT GCC --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---F Q R I T L Y L K E K K Y S P C A TGG GAG GTT GTC AGA GCA GAA ATC ATG AGA TCT TTT TCT TTG TCA ACA AAC TTG --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---W E V V R A E I M R S F S L S T N L CAA GAA AGT TTA AGA AGT AAG GAA TGA 3' --- --- --- --- --- --- ---ESLRSKE \*

# 20 Interferon Alpha Coding Sequence with All Codons Optimized (SEQ ID NO:11)

PCT/US99/05394

53

GCC GTG CGG AAG TAC TTC CAG CGG ATC ACC CTG TAC CTG AAG GAG AAG TAC TCC CCC

TGC GCC TGG GAG GTG GTG CGG GCC GAG ATC ATG CGG AGC TTC AGC CTG AGC ACC AAC CTG

5

CAG GAG AGC CTG CGG AGC AAG GAG TGA

# Additional/Semi Optimized Sequence Coding For Interferon Alpha (Second Line = SEQ ID NO:12)

10										10	0									20
	MET	ALA	LEU	THR	PHE	ALA	LEU	LEU	VAL	ALA	LEU	LEU	VAL	LEU	SER	CYS	LYS	SER	SER	CYS
	ATG	GCN	YTN	ACN	TTY	GCN	YTN	YTN	GTN	GCN	YTN	YTN	GTN	YTN	WSN	TGY	AAR	WSN	WSN	TGY
		<u></u>																		
	ATG	GCT	TTA	ACT	TTT	GCT	TTA	TTA	GTT	GCT	TTA	TTA	GTT	TTA	TCT	TGT	AAA	TCT	TCT	TGT
15		GCC	TTG	ACC	TTC	GCC	TTG	TTG	GTC	GCC	TTG	TTG	GTC	ŤTG	TCC	TGC	AAG	TCC	TCC	TGC
		GCA	CTT	ACA		GCA	CTT	CTT	GTA	GCA	CTT	CTT	GTA	CTT	TCA			TCA	TCA	
		GCG	CTC	ACG		GCG	CTC	CTC	GTG	GCG	CTC	CTC	GTG	CTC	TCG			TCG	TCG	
			CTA				CTA	СТА			CTA	CTA		CTA	AGT			AGT	AGT	
			CTG				CTG	CTG			CTG	CTG		CTG	AGC			AGC	AGC	
20																				
										30										40
	SER	VAL	GLY	CYS	ASP	LEU	PRO	GLN	THR	HIS	SER	LEU	GLY	SER	ARG	ARG	THR	LEU	MET	LEU
	WSN	GTN	GGN	TGY	GAY	YTN	CCN	CAR	ACN	CAY	WSN	YTN	GGN	WSN	MGN	MGN	ACN	YTN	ATG	YTN
•																				
25	TCT	GT <b>T</b>	GGT	TGT	GAT	TTA	CCT	CAA	ACT	CAT	TCT	TTA	GGT	TCT	CGT	CGT	ACT	TTA	ATG	TTA
	TCC	GTC	GGC	TGC	GAC	TTG	CCC	CAG	ACC	CAC	TCC	TTG	GGC	TCC	CGC	CGC	ACC	TTG		TTG
	TCA	GTA	GGA			CTT	CCA		ACA		TCA	CTT	GGA	TCA	CGA	CGA	ACA	CTT		CTT
	TCG	GTG	GGG			CTC	CCG		ACG		TCG	CTC	GGG	TCG	CGG	CGG	ACG	CTC		CTC
	AGT					CTA					AGT	CTA		AGT	AGA	AGA		CTA		CTA
30	AGC		•			CTG					AGC	CTG		AGC	AGG	AGG		CTG		CTG

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	CT	T G	CA			CGA	CGA	ATA	TCA	CTI	r	T			CTI			.c c.		AC	GAC	TT	
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										110										120
	GLU	THR	LEU	LEU	ASP	LYS	PHE	TYR	THR	GLU	LEU	TYR	GLN	GLN	LEU	ASN	ASP	LEU	GLU	ALA
	GAR	ACN	YTN	YTN	GAY	AAR	TTY	TAY	ACN	GAR	YTN	TAY	CAR	CAR	YTN	AAY	GAY	YTN	GAR	GCN
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5	GAA	ACT	TTA	TTA	GAT	AAA	TTT	TAT	ACT	GAA	TTA	TAT	CAA	CAA	TTA	AAT	GAT	TTA	GAA	GCT
	GAG	ACC	TTG	TTG	GAC	AAG	TTC	TAC	ACC	GAG	TTG	TAC	CAG	CAG	TTG	AAC	GAC	TTG	GAG	GCC
		ACA	CTT	CTT					ACA		CTT				CTT			CTT		GCA
		ACG	CTC	CTC					ACG		CTC				CTC			CTC		GCG
			CTA	CTA							CTA				CTA			CTA		
10			CTG	CTG							CTG				CTG			CTG		
										130					2					140
	CYS	VAL	ILE	GLN	GLY	VAL	GLY	VAL	THR	GLU	THR	PRO	LEU	MET	LYS	GLU	ASP	SER	ILE	LEU
	TGY	GTN	ATH	CAR	GGN	GTN	GGN	GTN	ACN	GAR	ACN	CCN	YTN	ATG	AAR	GAR	GAY	WSN	ATH	YTN
15																				<b></b>
	TGT	GTT	ATT	CAA	GGT	GTT	GGT	GTT	ACT	GAA	ACT	CCT	TTA	ATG	AAA	GAA	GAT	TCT	TTA	TTA
	TGC	GTC	ATC	CAG	GGC	GTC	GGC	GTC	ACC	GAG	ACC	CCC	TTG		AAG	GAG	GAC	TCC	ATC	TTG
		GTA	ATA		GGA	GTA	GGA	GTA	ACA		ACA	CCA	CTT					TCA	ATA	CTT
		GTG			GGG	GTG	GGG	GTG	ACG		ACG	CCG	CTC					TCG		CTC
20													CTA					AGT		CTA
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										150										160
25											LEU									
25	GCN	GTN	MGN	AAR	TAY	TTY	CAR	MGN	ATH	ACN	YTN	TAY	YTN	AAR	GAR	AAR	AAR	TAY	WSN	CCN
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				AAG	TAC	TTC					TTG			AAG	GAG	AAG	AAG	TAC		
30		GTA									CTT		CTT						TCA	
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			AGA					AGA			CTA		CTA						AGT	
			AGG					AGG			CTG		CTG						AGC	

			•							170										180
	CYS	ALA	TRP	GLU	VAL	VAL	ARG	ALA	GLU	ILE	MET	ARG	SER	PHE	SER	LEU	SER	THR	ASN	LEU
	TGY	GCN	TGG	GAR	GTN	GTN	MGN	GCN	GAR	ATH	ATG	MGN	WSN	TTY	WSN	YTN	WSN	ACN	AAY	YTN
5	TGT	GCT	TGG	GAA	GTT	GTT	CGT	GCT	GAA	ATT	ATG	CGT	TCT	TTT	TCT	TTA	TCT	ACT	AAT	TTA
	TGC	GCC		GAG	GTC	GTC	CGC	GCC	GAG	ATC		CGC	TCC	TTC	TCC	TTG	TCC	ACC	AAC	TTG
		GCA			GTA	GTA	CGA	GCA		ATA		CGA	TCA		TCA	CTT	TCA	ACA		СТТ
		GCG			GTG	GTG	CGG	GCG				CGG	TCG		TCG	CTC	TCG	ACG		CTC
							AGA					AGA	AGT		AGT	CTA	AGT			CTA
10							AGG					AGG	AGC		AGC	CTG	AGC			CTG

GLN GLU SER LEU ARG SER LYS GLU \*\*\*

CAR GAR WSN YTN MGN WSN AAR GAR TRR

15 --- --- --- --- --- --- --- --- --
CAA GAA TCT TTA CGT TCT AAA GAA TAA

CAG GAG TCC TTG CGC TCC AAG GAG TAG

TCA CTT CGA TCA TGA

TCG CTC CGG TCG

AGT CTA AGA AGT

AGC CTG AGG AGC

Delivery and expression of nucleic acids in many formulations is limited due to degradation of the nucleic acids by components of organisms, such as nucleases. Thus, protection of the nucleic acids when delivered in vivo can greatly enhance the resulting expression, thereby enhancing a desired pharmacological or therapeutic effect. It was found that certain types of compounds which interact with a nucleic acid (e.g., DNA) in solution but do not condense the nucleic acid provide in vivo protection to the nucleic acid, and correspondingly enhance the expression of an encoded gene product.

We have described the use of delivery systems designed to interact with plasmids and protect plasmids from rapid extracellular nuclease degradation [Mumper, R.J., et al.,

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1996, Pharm. Res. 13:701-709; Mumper, R.J., et al., 1997. Submitted to Gene Therapy]. A characteristic of the PINC systems is that they are non-condensing systems that allow the plasmid to maintain flexibility and diffuse freely throughout the muscle while being protected from nuclease degradation. While the PINC systems are primarily discussed below, it will be understood that cationic lipid based systems and systems utilizing both PINCS and cationic lipids are also within the scope of the present invention.

A common structural component of the PINC systems is 10 they are amphiphilic molecules, having hydrophilic and a hydrophobic portion. The hydrophilic portion of the PINC is meant to interact with plasmids by hydrogen bonding (via hydrogen bond acceptor or 15 groups), der Waals interactions, Van or/and by For example, PVP and N-methyl-2-pyrrolidone interactions. (NM2P) are hydrogen bond acceptors while PVA and PG are hydrogen bond donors.

All four molecules have been reported to form complexes 20 with various (poly)anionic molecules [Buhler V., Aktiengescellschaft Feinchemie, Ludwigshafen, рp 39-42; Galaev Y, et al., J. Chrom. A. 684:45-54 (1994); Tarantino R, et al. J. Pharm. Sci. 83:1213-1216 (1994); Zia, H., et Res. 8:502-504 (1991);]. al., Pharm. The hydrophobic portion of the PINC systems is designed to result in a 25 coating on the plasmid rendering its surface hydrophobic. Kabanov et al. have described previously the cationic polyvinyl derivatives for plasmid condensation designed to increase plasmid hydrophobicity, protect plasmid from nuclease degradation, and increase its 30 affinity for biological membranes [Kabanov, A.V., Kabanov, V.A., 1995, Bioconj. Chem. 6:7-20; Kabanov, A.V., et al., 1991, Biopolymers 31:1437-1443; Yaroslavov, A.A., et al., 1996, FEBS Letters 384:177-180].

35 Substantial protective effect is observed; up to at least a one log enhancement of gene expression in rat muscle

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over plasmid formulated in saline has been demonstrated with these exemplary PINC systems. We have also found that the expression of reporter genes in muscle using plasmids complexed with the PINC systems was more reproducible than when the plasmid was formulated in saline. For example, the coefficient of variation for reporter gene expression in muscle using plasmid formulated in saline was 96  $\pm$  35% (n = 20 studies; 8-12 muscles/study) whereas with coefficient of variation with plasmids complexed with PINC systems was 40  $\pm$ 30 studies; 8-12 muscles/study). The high coefficient of variation for reporter gene expression with plasmid formulated in saline has been described previously [Davis, H.L., et al., 1993, Hum. Gene Ther. 4:151-9]. addition, in contrast with the results for DNA: saline, there was no significant difference in gene expression in muscle when plasmid with different topologies were complexed with polyvinyl pyrrolidone (PVP). This suggests that PVP is able to protect all forms of the plasmid from rapid nuclease degradation.

# 20 1. Summary of interactions between a PINC polymer (PVP) and plasmid

We have demonstrated using molecular modeling that an exemplary PINC polymer, PVP, forms hydrogen bonds with the base pairs of a plasmid within its major groove and results in a hydrophobic surface on the plasmid due to the vinyl backbone of PVP. These interactions are supported by the modulation of plasmid zeta potential by PVP as well as by inhibition of ethidium bromide intercalation into complexed plasmid. We have correlated apparent binding between PVP and plasmid to pH and salt concentration and have demonstrated the effect of these parameters on  $\square$ -gal expression after intramuscular injection of plasmid/PVP complexes [Mumper, R.J., et al., 1997. Submitted to Gene A summary of the physico-chemical properties of Therapy]. plasmid/PVP complexes is listed in Table I below.

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Table I: Summary of the Physico-Chemical Properties of Plasmid/PVP Complexes

Method Result Molecular modeling Hydrogen bonding and Fourier-transformed hydrophobic plasmid surface Infra-red Hydrogen observed bonding demonstrated DNase I challenge Decreased rate of plasmid degradation in the presence of PVP Microtitration Positive heats of reaction Calorimetry indicative of an endothermic process Potentiometric titration One unit pH drop when plasmid and PVP are complexed Dynamic Dialysis Rate of diffusion of PVP reduced in the presence of plasmid Zeta potential Surface charge of plasmid modulation decreased by PVP Ethidium bromide Ethidium bromide Intercalation intercalation reduced by plasmid/PVP complexation Osmotic pressure Hyper-osmotic formulation (i.e., 340  $mOsm/kg H_2O$ ) Luminescence Plasmid/PVP binding decreased Spectroscopy in salt and/or at pH 7

## 5 2. <u>Histology of expression in muscle</u>

Immunohistochemistry for  $\beta$ -gal using a slide scanning technology has revealed the uniform distribution of  $\beta$ -gal expression sites across the whole cross-sections of rat tibialis muscles. Very localized areas were stained positive for  $\beta$ -gal when CMV- $\beta$ -gal plasmid was formulated in saline.  $\beta$ -gal positive cells were observed exclusively around the needle tract when plasmid was injected in saline. This is

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in agreement with previously published results [Wolff, J.A., et al., 1990, Science 247:1465-68; Davis, H.L., et al., 1993, Hum. Gene Ther. 4:151-9; Davis, H.L., et al., 1993, Hum. Gene Ther. 4:733-401.

In comparison, immunoreactivity for  $\beta\text{-gal}$  was observed 5 wide area of muscle tissue after intramuscular injection of CMV- $\beta$ -gal plasmid/PVP complex (1:17 w/w) in 150 It appeared that the majority of positive muscle fibers were located at the edge of muscle bundles. Thus, staining for  $\beta\text{-gal}$  in rat muscle demonstrated that, using a 10 plasmid/PVP complex, the number of muscle fibers stained positive for  $\beta$ -gal was approximately 8-fold greater than found using a saline formulation. Positively stained muscle fibers were also observed over a much larger area in the muscle tissue using the plasmid/PVP complex providing evidence that the injected plasmid was widely dispersed after intramuscular injection.

We conclude that the enhanced plasmid distribution and expression in rat skeletal muscle was a result of both protection from extracellular nuclease degradation due to complexation and hyper-osmotic effects of the plasmid/PVP However, Dowty and Wolff et al. have 'demonstrated that osmolarity, up to twice physiologic osmolarity, did not significantly effect gene expression in muscle [Dowty, M.E., Wolff, and J.A. In: J.A. Wolff (Ed.), 1994, Gene Therapeutics: Methods and Applications of Direct Transfer. Birkhauser, Boston, pp. 82-98]. This suggests the enhanced expression of due plasmid PVP complexation is most likely due to nuclease protection and less to osmotic effects. Further, the surface modification of plasmids by PVP (e.g., increased hydrophobicity decreased negative surface charge) may also facilitate the uptake of plasmids by muscle cells.

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#### Structure-activity relationship of PINC 3. polymers

We have found a linear relationship between structure of a series of co-polymers of vinyl pyrrolidone and vinyl acetate and the levels of gene expression in rat We have found that the substitution of some vinyl pyrrolidone monomers with vinyl acetate monomers in PVP resulted in a co-polymer with reduced ability to form The reduced interaction hydrogen bonds with plasmids. subsequently led to decreased levels of gene expression in rat muscle after intramuscular injection. The expression of  $\beta$ -gal decreased linearly (R = 0.97) as the extent of vinyl content in the co-polymers (VPM) pvrrolidone monomer decreased.

These data demonstrate that pH and viscosity are not the most important parameters effecting delivery of plasmid to muscle cells since these values were equivalent for all These data suggest that enhanced binding of the PINC polymers to plasmid results in increased protection and bioavailability of plasmid in muscle. 20

#### Additional PINC systems

The structure-activity relationship described above can be used to design novel co-polymers that will also have It is expected that enhanced interaction with plasmids. there is "an interactive window of opportunity" whereby enhanced binding affinity of the PINC systems will result in gene expression after enhancement of further intramuscular injection due to more extensive protection of It is expected that plasmids from nuclease degradation. there will be an optimal interaction beyond which either condensation of plasmids will occur or "triplex" type either of which can result in bioavailability in muscle and consequently reduced gene expression.

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As indicated above, the PINC compounds are generally amphiphilic compounds having both a hydrophobic portion and a hydrophilic portion. In many cases the hydrophilic portion is provided by a polar group. It is recognized in the art that such polar groups can be provided by groups such as, but not limited to, pyrrolidone, alcohol, acetate, amine or heterocyclic groups such as those shown on pp. 2-73 and 2-74 of CRC Handbook of Chemistry and Physics (72nd Edition), David R. Lide, editor, including pyrroles, 10 pyrazoles, imidazoles, triazoles, dithiols, oxazoles, (iso)thiazoles, oxadiazoles, oxatriazoles, diaoxazoles, oxathioles, pyrones, dioxins, pyridines, pyridazines, pyrimidines, pyrazines, piperazines, (iso)oxazines, indoles, indazoles, carpazoles, and purines and derivatives of these groups, hereby incorporated by reference. 15

Compounds also contain hydrophobic groups which, in the case of a polymer, are typically contained in the backbone of the molecule, but which may also be part of a nonpolymeric molecule. Examples of such hydrophobic backbone groups include, but are not limited to, vinyls, 20 acrylates, acrylamides, esters, celluloses, hydrides, ethers, carbonates, phosphazenes, sulfones, propylenes, and derivatives of these groups. The polarity characteristics of various groups are quite well known to those skilled in the art as illustrated, for example, by 25 discussions of polarity in any introductory chemistry textbook.

The ability of such molecules to interact with nucleic acids is also understood by those skilled in the art, and can be predicted by the use of computer programs which model 30 such intermolecular interactions. Alternatively or addition to such modeling, effective compounds can readily identified using one or more of such tests as determination of inhibition of the rate of nuclease digestion, 2) alteration of the zeta potential of the DNA, 35 which indicates coating of DNA, 3) or inhibition of the

ability of intercalating agents, such as ethidium bromide to intercalate with DNA.

#### Targeting Ligands

In addition to the nucleic acid/PINC complexes described above for delivery and expression of nucleic acid sequences, in particular embodiments it is also useful to provide a targeting ligand in order to preferentially obtain expression in particular tissues, cells, or cellular regions or compartments.

Such a targeted PINC complex includes a PINC system (monomeric or polymeric PINC compound) complexed to plasmid (or other nucleic acid molecule). The PINC system is covalently or non-covalently attached to (bound to) a targeting ligand (TL) which binds to receptors having an affinity for the ligand. Such receptors may be on the surface or within compartments of a cell. Such targeting provides enhanced uptake or intracellular trafficking of the nucleic acid.

The targeting ligand may include, but is not limited 20 to, galactosyl residues, fucosal residues, mannosyl residues, derivatives, monoclonal carnitine antibodies, polyclonal antibodies, peptide ligands, and DNA-binding proteins. Examples of cells which may usefully be targeted include, but are not limited to, antigen-presenting cells, 25 hepatocytes, myocytes, epithelial cells, endothelial cells, and cancer cells.

Formation of such a targeted complex is illustrated by the following example of covalently attached targeting ligand (TL) to PINC system:

TL-PINC + Plasmid -----> TL-PINC:::::Plasmid
Formation of such a targeted complex is also
illustrated by the following example of non-covalently
attached targeting ligand (TL) to PINC system
TL:::::PINC + Plasmid -----> TL:::::PINC:::::Plasmid
or alternatively,

PINC + Plasmid -----> PINC:::::Plasmid + TL ------> TL:::::PINC:::::Plasmid

In these examples :::::: is non-covalent interaction such as ionic, hydrogen-bonding, Van der Waals interaction,

5 hydrophobic interaction, or combinations of such interactions.

A targeting method for cytotoxic agents is described in Subramanian et al., International Application PCT/US96/08852, International Publication No. WO 96/39124, 10 hereby incorporated by reference. This application describes the use of polymer affinity systems for targeting cytotoxic materials using a two-step targeting involving zip polymers, i.e., pairs of interacting polymers. An antibody attached to one of the interacting polymers 15 binds to a cellular target. That polymer then acts as a target for a second polymer attached to a cytotoxic agent. As referenced in Subramanian et al., other two-step (or multi-step) systems for delivery of toxic agents are also described.

20 In another aspect, nucleic acid coding sequences can be delivered and expressed using a two-step targeting approach involving a non-natural target for a PINC system or PINCtargeting ligand complex. Thus, for example, a PINC-plasmid complex can target a binding pair member which is itself attached to a ligand which binds to a cellular target (e.g., 25 Binding pairs for certain of a MAB). the compounds identified herein as PINC compounds as identified Subramanian et al. Alternatively, the PINC can be complexed to a tareting ligand, such as an antibody. That antibody 30 can be targeted to a non-natural target which binds to, for example, a second antibody.

# III. Model Systems for Evaluation of Interferon Alpha Constructs and Formulations

In accord with the concept of using interferon alpha 35 expressing plasmid constructs and formulations in anti-

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cancer treatment, murine model systems were utilized based on murine tumor cell lines. The line primarily used was S.C. VII/SF, which is a cell line derived from murine squamous cell carcinoma (S.C.).

Squamous cell carcinoma of the head and neck begins with the cells lining the oral and pharyngeal cavities. Clinical disease progresses via infiltration and spreads underlying tissues and lymphatics. The into the undifferentiated, in vivo passage tumor line S.C. VII/SF displays this typical growth pattern. In addition, rapid growth rate provides a relatively short test period for individual experiments. Other murine tumor cell lines include another SCC line KLN-205, a keratinocyte line I-7, and a colon adenocarcinoma line MC-38.

An optimal model system preferably satisfies the criteria based on having tumor growth rate in vivo (i.e., tumors are ready for treatment in 4-10 days post implant), invasiveness, and local spread similar to those observed in clinical disease, and providing accessibility for experimental treatment. As indicated, the SCC VII/SF cell line was utilized as the primary model system cell line. This cell line typically grows rapidly, resulting in death of untreated syngeneic mice 14-17 days after tumor cell implantation.

25 This cell line can be utilized in a variety of ways to provide model system suitable for a variety of different tests. Four such possibilities are described below.

First, SCCVII cells can be utilized in cell culture to provide an *in vitro* evaluation of interferon alpha expression construct and formulation characteristics, such as expression levels and cellular toxicities.

Second, the cells can be implanted subcutaneously in mice. This system can be utilized in tests in which accessibility of the implant site is beneficial. As an example, the method was utilized in evaluations of

expression efficiencies based on the expression of chloramphenical acetyltransferase (CAT).

Third, the cells can be implanted transcutaneously into the fascia of digastric muscle.

Fourth, the cells can be implanted transcutaneously into digrastric/mylohyoid muscles. The important features of models 3 and 4 are shown in the table below.

TABLE II: Comparison of submandibular tumor models

Feature	Mouse Tumor Model 3	Mouse Tumor Model 4
Tumor implant procedure	2-4 x 10 <sup>5</sup> cells	5 x 10 <sup>5</sup>
r-vocaure	transcutaneously into	transcutaneously into digastric/mylohyoid
Tumor growth and invasiveness characteristics	Prominent submandibular bulge; invasion of digastric/mylohyoid muscles and	muscles  More variable, invasion of digastric/mylohyoid muscles and lymphatics
Treatment procedure (primary treatment)	lymphatics Transcutaneous, needle inserted and moved within tumor to produce a 4 quadrant distribution of gene medicine	Lower jaw skin flap raised to expose tumor, needle inserted and moved within tumor to produce a 4 quadrant distribution
Days treated (post-implant)	Day 5, day 10 (both transcutaneously)	of gene medicine  Day 5 (tumor exposed),  day 8
Measurement procedure	External calipering 2-3 x per week until death	(transcutaneously)  First caliper when tumor exposed for treatment, second caliper at sacrifice

Feature	Mouse Tumor Model 3	Mouse Tumor Model 4
Advantages	Non-surgical, closed	Surgical, open model
	model allows larger	allows direct
	experiments and more	treatment of exposed
	frequent treatments;	tumor; Local
	Sacrifice unnecessary	inflammation from
	to caliper (=more	surgery may
	time points)	additionally stimulate
		immune response; More
		like clinical
		situation for protocol
		development
Disadvantages	Transcutaneous	Labor intensive;
	treatment is	Smaller, fewer
	potentially less	experiments possible;
	accurate and	Tumors deeper and more
	intensive; less like	difficult to treat
	expected clinical	transcutaneously (for
	treatments than	secondary treatments);
	surgical approaches	Fewer treatments and
		caliperings possible

The tumor size treated in the mouse models is generally 20-50 mm<sup>3</sup>. A 50 mm<sup>3</sup> mouse tumor is approximately equivalent to 150 cc<sup>3</sup> human tumor having an average diameter of about 6.6 cm. This tumor size is approximately 10-fold larger than the size proposed to be treated in the phase I clinical trials. This indicates that the mouse models are strongly biased towards over estimating the expected tumor burden in human patients.

#### IV. Formulations for In Vivo Delivery

#### A. General

While expression systems such as those described above provide the potential for expression when delivered to an appropriate location, it is beneficial to provide the expression system construct(s) in a delivery system which

can assist both the delivery and the cellular uptake of the construct. Thus, this invention also provides particular formulations which include one or more expression system constructs (e.g., DNA plasmids as described above), and a protective, interactive non-condensing compound.

An additional significant factor relating to the plasmid construct is the percentage of plasmids which are in a supercoiled (SC) form rather than the open circular (OC) form.

### 10 B. <u>Delivery and Expression</u>

A variety of delivery methods can be used with the constructs and formulations described above, in particular, delivery by injection to the site of a tumor can be used. The submandibular tumor models utilized injection into four quadrants of the tumor being treated.

# C. Anti-Cancer Efficacy of Human Interferon Alpha Formulations

The effects of the administration of the interferon alpha formulations described above were evaluated using the S.C. VII mouse tumor models. Plasmid constructs as described above were incorporated in delivery formulations. The formulations were delivered by injection.

# D. Synergistic Effects of Interferon Alpha plasmid and IL-12 Plasmid and Effect of Human Interferon Alpha Formulation Administration on Production of Secondary Cytokines

The effects of the expression of the human interferon alpha plasmids in tumor cells on the progress of the mouse tumors demonstrates that such interferon alpha expression is effective against such tumors. However, it was also shown that IL-12 can act synergistically with the interferon alpha expression to exercise the antitumor effect (see Figure 9).

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## E. Toxicity Evaluation of Exemplary Formulations

The exemplary formulations do not show high cellular toxicity at the concentrations tested, suggesting that the formulations do not significantly kill cells by direct toxic action in vivo. Moreover, the anti-tumor activity induced by IFN $\alpha$  gene therapy is dependent upon activation of the immune system, which is demonstrated by depletion studies in vivo. Removal of a specific T lymphocyte population (CD8 $^+$ ) abrogates the anti-tumor activity elicited by IFN $\alpha$  gene therapy.

#### V. Administration

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Administration as used herein refers to the route of introduction of a plasmid or carrier of DNA into the body. In addition to the methods of delivery described above, the expression systems constructs and the delivery system formulations can be administered by a variety of different methods.

Administration can be directly to a target tissue of by targeted delivery to the target tissue after systemic administration. In particular, the present invention can be used for treating disease by administration of the expression system or formulation to the body in order to establishing controlled expression of any specific nucleic acid sequence within tissues at certain levels that are useful for gene therapy.

The preferred means for administration of vector (plasmid) and use of formulations for delivery are described above. The preferred embodiments are by direct injection using needle injection.

The route of administration of any selected vector construct will depend on the particular use for the expression vectors. In general, a specific formulation for each vector construct used will focus on vector uptake with regard to the particular targeted tissue, followed by demonstration of efficacy. Uptake studies will include

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uptake assays to evaluate cellular uptake of the vectors and expression of the DNA of choice. Such assays will also determine the localization of the target DNA after uptake, and establishing the requirements for maintenance of steadystate concentrations of expressed protein. cytotoxicity can then be tested. Toxicity will not only include cell viability but also cell function.

Muscle cells have the unique ability to take up DNA from the extracellular space after simple injection of DNA particles as a solution, suspension, or colloid into the Expression of DNA by this method can be sustained muscle. for several months.

Delivery of formulated DNA vectors involves incorporating DNA into macromolecular complexes that undergo endocytosis by the target cell. 15 Such complexes may include lipids, proteins, carbohydrates, synthetic compounds, or inorganic compounds. Preferably, the complex includes DNA, a cationic lipid, and a neutral lipid in particular proportions. The characteristics of the complex 20 formed with the vector (size, charge, surface characteristics, composition) determines the bioavailability of the vector within the body. Other elements of the formulation function as ligand which interact with specific receptors on the surface or interior of the cell. Other elements of the formulation function to enhance entry into the cell, release from the endosome, and entry into the nucleus.

Delivery can also be through use of DNA transporters. DNA transporters refers to molecules which bind to vectors and are capable of being taken up by epidermal cells. DNA transporters contain a molecular complex capable of noncovalently binding to DNA and efficiently transporting the DNA through the cell membrane. It is preferable that the transporter also transport the DNA through the nuclear membrane. See, e.g., the following applications all which (including drawings) are hereby incorporated by reference herein: (1)Woo et al., U.S. Serial

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07/855,389, entitled "A DNA Transporter System and Method of Use,, filed March 20, 1992, now abandoned; (2) Woo et al., PCT/US93/02725, International Publ. WO93/18759, entitled "A DNA Transporter System and Method of Use", (designating the other countries) filed March 19, continuation-in-part application by Woo et al., entitled "Nucleic Acid Transporter Systems and Methods of Use", filed December 14, 1993, U.S. Serial No. 08/167,641; (4) Szoka et al. , U.S. Serial No. 07/913,669, entitled "Self-Assembling Polynucleotide Delivery System", filed July 14, 1992 and (5) 10 Szoka et al., PCT/US93/03406, International Publ. WO93/19768 entitled "Self-Assembling Polynucleotide Delivery System", (designating the U.S. and other countries) filed April 5, A DNA transporter system can consist of particles containing several elements that are independently and non-15 covalently bound to DNA. Each element consists of a ligand which recognizes specific receptors or other functional groups such as a protein complexed with a cationic group that binds to DNA. Examples of cations which may be used 20 spermine, spermine derivatives, histone, cationic peptides and/or polylysine. One element is capable of binding both to the DNA vector and to a cell surface receptor on the target cell. Examples of such elements are organic compounds which interact with the asialoglycoprotein 25 receptor, the folate receptor, the mannose-6-phosphate receptor, or the carnitine receptor. A second element is capable of binding both to the DNA vector and to a receptor on the nuclear membrane. The nuclear ligand is capable of recognizing and transporting a transporter system through a 30 nuclear membrane. An example of such ligand is the nuclear targeting sequence from SV40 large T antigen or histone. A third element is capable of binding to both the DNA vector and to elements which induce episomal lysis. include inactivated virus particles such as adenovirus, 35 peptides related to influenza virus hemagglutinin, or the GALA peptide described in the Szoka patent cited above.

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Transfer of genes directly into a tumor has been very effective. Experiments show that administration by direct injection of DNA into tumor cells results in expression of the gene in the area of injection. Injection of plasmids containing human interferon alpha results in expression of the gene for 5 days following a single intra-tumoral injection. Human IFN $\alpha$  production was highest in tumors harvested 1 day post-tumor injection and steadily declined thereafter. The injected DNA appears to persist in an unintegrated extrachromosomal state. This means of transfer is a preferred embodiment.

Administration may also involve lipids as described in preferred embodiments above. The lipids may form liposomes which are hollow spherical vesicles composed of arranged in unilamellar, bilamellar, or multilamellar fashion and an internal aqueous space for entrapping water soluble compounds, such as DNA, ranging in size from 0.05 to several microns in diameter. Lipids may be useful without forming liposomes. Specific examples include the use of cationic lipids and complexes containing DOPE which interact DNA and with the membrane of the target cell to facilitate entry of DNA into the cell.

Gene delivery can also be performed by transplanting genetically engineered cells. For example, immature muscle cells called myoblasts may be used to carry genes into the muscle fibers. Myoblast genetically engineered to express recombinant human growth hormone can secrete the growth hormone into the animal's blood. Secretion of the incorporated gene can be sustained over periods up to 3 months.

Myoblasts eventually differentiate and fuse to existing muscle tissue. Because the cell is incorporated into an existing structure, it is not just tolerated but nurtured. Myoblasts can easily be obtained by taking muscle tissue from an individual who needs gene therapy and the genetically engineered cells can also be easily put back with out causing damage to the patient's muscle. Similarly,

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keratinocytes may be used to delivery genes to tissues. numbers of keratinocytes can be generated cultivation of a small biopsy. The cultures can be prepared as stratified sheets and when grafted to humans, generate epidermis which continues to improve in histotypic quality many The keratinocytes are years. genetically engineered while in culture by transfecting Although keratinocytes with the appropriate vector. keratinocytes are separated from the circulation by the basement membrane dividing the epidermis from the dermis, human keratinocytes secrete into circulation the protein produced.

chosen method of delivery should result expression of the gene product encoded within the nucleic cassette at levels which exert appropriate an biological effect. The rate of expression will depend upon the disease, the pharmacokinetics of the vector and gene product, and the route of administration, but should be in range 0.001-100 mg/kg of body weight /day, preferably 0.01-10 mg/kg of body weight/day. This level is readily determinable by standard methods. It could be more or less depending on the optimal dosing. The duration of treatment will extend through the course of the disease symptoms, possibly continuously. The number of doses will depend upon the disease, delivery vehicle, and efficacy data from clinical trials.

#### Examples

The present invention will be more fully described in conjunction with the following specific examples which are not to be construed in any way as limiting the scope of the invention. As shown below, mIFN- gene medicine reduces the growth of tumors in syngeneic murine tumor models. Lipid formulations of mIFN- gene medicine display anti tumor activity in both SCC-VII and MC-38 tumor models. PINC and peptide formulations of mIFN- gene medicine display anti

tumor effects in the MC-38 tumor model. The anti tumor effects of mIFN- gene medicine are dose dependent. In addition, the examples demonstrate that treatment of tumors with the combination of IFN $\alpha$  and IL-12 gives an unanticipated more than additive (synergystic) anti-tumor activity using either a PINC or a lipid formulation.

#### Example 1

A plasmid expression system encoding murine IFNlpha4 and formulated in a polymeric delivery system was used for in 10 immunotherapeutic activity against an immunogenic murine renal cell carcinoma, Renca, and a non-immunogenic mammary adenocarcinoma, TS/A. Mice bearing established tumors were treated with IFN $\alpha$ /polyvinyl-pyrrolidone (PVP) expression complexes via direct intra-tumoral injection. to 100 % tumor growth inhibition was observed in the treated 15 By using an optimal dose of 96 and 48  $\mu g$  of formulated IFN- $\alpha$  plasmid for the treatment of Renca and TS/A respectively, 30% (Renca) and 10% (TS/A) of the treated animals remained tumor-free. Tumor inhibition was dependent 20 upon activation of the immune system. The anti-tumor activity elicited by IFN-lpha gene therapy was abrogated when mice were selectively depleted of CD8<sup>+</sup> T cells. By contrast, removal of CD4<sup>+</sup> resulted in increased tumor rejection following IFN- $\alpha$ /PVP treatments. Finally, mice that remained 25 tumor-free following IFN- $\alpha$  gene therapy displayed immune resistance to a subsequent challenge of tumor. These data provide evidence that non-viral IFN $\alpha$  gene therapy can be used to induce an efficient anti-tumor response.

Local presence of cytokines in tumors can activate an immune response that in some cases leads to induction of specific long-lasting anti-tumor immunity. By direct intratumoral injection of plasmid encoding murine IFN $\alpha 4$  and formulated in a polymeric delivery system, tumor-bearing mice develop an immune response, which leads to inhibition and eradication of the tumor. We have shown by depletion

studies in vivo that the immune response induced by IFN $\alpha$  is mainly CD8-mediated and that this treatment results in a long-term immunity in mice demonstrating complete tumor regression. Thus, non-viral IFN $\alpha$  gene therapy may be an effective alternative to IFN $\alpha$  protein therapy for human cancers.

Transduction of tumor cells with cytokine genes has proven to be a very efficient technique to induce cytokinemediated anti-tumor immunity. In experimental models, the 10 local presence of IL-2, IL-1, IL-4, IL-6, IL-7, IL-12, IFNs and CSFs (i.e., GM-CSF) at the site of the tumor can result in significant tumor growth inhibition: (Colombo et al., "Local Cytokine Availability Elicits Tumor Rejection and Systemic Immunity Through Granulocyte-T-Lymphocyte Cross-Talk", Cancer Research, 52, 4853-4857 (1992)). 15 systems, cytokines have limited effect on proliferation directly but are capable of activating a rapid and potent anti-tumor immune response, which impedes tumor progression. Established parental tumors, however, 20 difficult to eradicate with ex vivo cytokine-transduced tumor cells because efficacy of vaccination is highly dependent on the size, growth rate and invasiveness of the tumor.

To overcome these problems, cytokine-based gene therapy approaches, which can deliver transgenic cytokines locally and induce an anti-tumor immune response, have been recently evaluated by a number of investigators (Forni et "Cytokine-Induced Immunogenicity: From Exogenous Cytokines to Gene Therapy", Journal of Immunotherapy, 253-257, 14, (1993); Pericle et al., "An Efficient Th2-type Memory Follows Cd8+ Lymphocyte-driven and Eosinophil-mediated Rejection of а Spontaneous Mouse Mamary Adenocarcicoma Engineered to Release Il-4", The Journal of Immunology, 153, 5660-5673. (1994); Pardoll et al., "Gene Modified Tumor Vaccines, In Cytokine-Induced Tumor Immunogenicity", eds. Academic Press, London, p. 71-86. (1994); and Musiani et

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al., "Cytokines, Tumor-cell Death and Immunogenicity: A Question of Choice", Immunology Today. 1, 32-36 (1997)). Technological breakthroughs in gene therapy adenoviral, retroviral, and liposomal vectors have provided powerful tools with which to study the biological effects of specific cytokine mediators as well as to develop novel and clinically applicable anti-tumor immunotherapies (Pardoll, "Paracrine Cytokine Adjuvants in Cancer Immunotherapy", Annu. Rev. Immunol. 13, 399-415 (1995); Bramson et al., "Direct Intratumoral Injection of an Adenovirus Expressing 10 Interleukin-12 Induces Regression and Long-lasting Immunity That Is Associated with Highly Localized Expression of Interleukin-12", Hum. Gene Ther., 7, 1995-2002 (1996); Rao et al., "I1-12 Is an Effective Adjuvant to Recombinant Vaccinia Virus-based Tumor Vaccines", J. Immunol. 15 3357-3365. 1996; Rakhmilevich et al., "Gene Gun-mediated Skin Transfection with Interleukin 12 Gene Results Regression of Established Primary and Metastatic Murine Tumors", Proc. Natl. Acad. Sci. USA. 93, 6291-6296 (1996); and Rakhmilevich et al, "Cytokine Gene Therapy of Cancer 20 Using Gene Gun Technology: Superior Antitumor Activity of Interleukin-12", Hum. Gene Ther. 8, 1303-1311, (1997)).

gene therapy approach utilizing an interactive polymeric gene delivery system that increases expression by protecting plasmid DNA (pDNA) from nucleases 25 and controlling the dispersion and retention of pDNA in muscle cells is described in Mumper et al., 1996. polymeric interactive non-condensing (PINC) systems routinely result in a greater amount of gene expression from tissues as compared to delivery of unformulated plasmid in 30 saline (Mumper et al., 1996). By using a plasmid that encodes human insulin growth factor-1 (hIGF-1) formulated as a PINC complex, production of biologically active h IGF-1 in vivo following intra-muscular injection has been shown (Alila et al., "Expression of Biologically 35 Active Human Insulin-Like Growth Factor-1 Following

Intramuscular Injection of a Formlated Plasmid in Rats", <u>Human Gene Therapy</u>, 8, 1785-1795 (1997)). The specific objective of this study was to determine whether a plasmid expression system encoding murine IFNa4 and formulated as a complex with PVP could induce an anti-tumor immune response following direct injection into subcutaneous murine tumors.

The IFN family consists of three major glycoproteins, IFNα, IFNβ and IFNγ. Although IFNs were first developed as antiviral agents, it is now clear that they also control growth and differentiation, and modulate various 10 aspects of host immunity (Gresser et al., "Antitumor effects of interferon", Acta Oncol. 28, 347-353 (1989)). Clinical data concluded that systemic chronic administration of  $\text{IFN}\alpha$ could produce regression of vascular tumors, including hemangiomastosis, 15 Kaposi's sarcoma, pulmonary (Singh et al., "Interferons A and B Downhemangiomas regulate the Expression of Basic Fibroblast Growth Factor in Human Carcicomas", Proc. Natl. Acad. Sci. USA. 92, 4562-4566 (1995)). Although IFN $\alpha$  was the first cytokine to be used in clinical trials that proved to be effective against certain 20 types of human cancer, only recently has this cytokine been considered as a candidate for gene therapy (Ogura et al. Belldegrun et al., "Human Renal Carcinoma Transfected With Interleukin-2 and/or Interferon  $\alpha$  Gene(s): Implications for Live Cancer Vaccines, Journal 25 National Cancer Institute, 85, 207-216 (1993).

studies have shown that the injection of Initial producing tumor cells IFΝα genetically modified syngeneic mice induces tumor growth inhibition and elicits a tumor-specific immune memory (Ferrantini et al., Interferon Alpha-1-Interferon Gene Transfer into Metastatic Friend Lukemia Cells Abrogated Tumorigenicity in Immunocompetent Mice: Antitumor Therapy by Means of Interferon-Producing Cells; Cancer Res. 53, 1107-4615 (1993); Ferrantini et al., Metastatic Murine Expression into а "Ifn-al Gene Adenocarcicoma (Ts/a) Results in Cd8+ T Cell-Mediated Tumor

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Rejection and Development of Antitumor Immunity: Comparative Studies with Ifn- $\gamma$ -producing Ts/a Cells" Journal of Immunology, 153, 4604-4615, (1994); Musiani et al. 1997). However, the real value of this potential form of vaccine in inducing the regression of established tumors remains to be demonstrated.

In this study we present evidence that direct injection of IFN $\alpha$  plasmid formulated in PVP into subcutaneous murine tumors results in a host-dependent tumor rejection, primarily mediated by CD8 $^+$  T cells, and elicits a protective immunity against subsequent tumor re-challenge.

# Materials And Methods

# Plasmid construction and formulation

A plasmid expression system containing an expression cassette for mIFN-l $\alpha 4$  was constructed as follows. 15 The coding sequence of the murine IFN- $\alpha4$  gene (Genebank X01973 M15456 M23830 X01967) was amplified by PCR from mouse genomic DNA. amplified mIFN- $\alpha4$  sequence was then subcloned into a plasmid backbone, and the sequence fidelity was verified by DNA sequence analysis (data not shown). The coding sequence for mIFN- $\alpha4$  was 20 then subcloned as an XbaI-BamHlfragment into the expression plasmid pIL0697 to create the mIFN- $\alpha4$  expression system pIF0836. Plasmid pVC0612 (empty plasmid, EP) contains expression elements including the cytomegalovirus immediate early promoter and the 3' UTR/poly(A) signal from the bovine growth gene in the pVC0289 25 backbone described by Alila et al. (1997). Plasmid pVC0612 was used as a control plasmid in all in vivo experiments. for intra-tumoral injection were grown under kanamycin selection in E. coli host strains DH5lpha and purified using conventional alkaline lysis and chromatographic methods. 30 Purified plasmid utilized intra-tumoral for injections had the following specifications: endotoxin (< 500 Eu/mg plasmid); protein (< 1%); and chromosomal DNA (< 20 %). Purified pIF0836 and control

plasmids were formulated at a concentration of 3 mg DNA/ml in a solution of 5 % w/v polyvinyl-pyrrolidone (Plasdone C-30, ISP Technologies, Wayne, NJ), 150 mM NaCl on the day of injection, as described previously (Mumper et al., 1996).

#### 5 Western blot analysis and bioassay for mIFNα.

HeLa cells were plated in 6 well plates at 3 x 10<sup>5</sup> cells per well, and transfected using 1 aµg of mouse IFNa4 plasmid pIF0836C and 3 µg of Lipofectamine (Life Technologies, Inc., Gaithersburg, MD) in serum-free DMEM. Same supernatants were harvested 24 hours later and immunoprecipitated using anti-mouse interferon  $\alpha/\beta$  polyclonal antibody (BioSource International, Camarillo, CA) and protein A and G agarose (Boehringer Mannheim, Indianapolis, IN). Samples were run on a 12% Tris-glycine gel and electroblotted to Millipore PVDF membrane. Anti-mouse interferon  $\alpha/\beta$  polyclonal antibody followed by anti-sheep 1:1000, Ιg used at 1:1000. Biotinylated molecular (Boehringer Mannheim) at detected using Streptavidin-HRP markers were (Amersham, Arlington Heights, IL). Detection was performed using the Amersham ECL kit. Supernatants were also tested for IFN $\alpha$  biological activity using L929 cells treated with encephalomyocarditis virus, in parallel with a NIH mouse IFNα reference reagent (Access Biomedical, San Diego, CA).

#### Animals

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Normal 8-week-old female BALB/c mice were purchased from Harlan Laboratories, Houston, TX. Mice were maintained on ad libitum rodent feed and water at 23°C, 40% humidity, and a 12-h/12-h light-dark cycle. Animals were acclimated for at least 4 days before the start of the study.

#### 30 Tumors

Three established mouse tumor models were used in this study. TS/A is a tumor cell line established by Dr. P. Nanni, University of Bologna, Italy, from the first in vivo

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transplant moderately differentiated of a mammary adenocarcinoma that spontaneously arose in a BALB/c mouse et al., A number of pre-immunization-1983). challenge experiments suggested that TS/A does not elicit a long-lasting anti-tumor immunity (Forni et al., 1987). was generously provided by Dr. Guido Forni, University of Turin, Italy. Renca, a spontaneously arising murine renal cell carcinoma, and CT-26, a colon adenocarcinoma, generously provided by Dr. Drew M. Pardoll, John Hopkins 10 Hospital, Baltimore, MD. Tumor cell cultures maintained in sterile disposable flasks from Corning (Corning, NY) at  $37^{\circ}$  C in a humidified  $5^{\frac{1}{6}}$  CO<sub>2</sub> atmosphere, using RPMI 1640 supplemented with 10% FBS, penicillin, 100 U/ml streptomycin and 50  $\mu$ g/ml gentamycin; all from Life Technologies.

# In vivo evaluation of tumor growth and treatments

BALB/c mice were challenged s.c. in the middle of the left flank with 30  $\mu l$  of a single-cell suspension contained the specified number of cells. Seven days later when the tumor size reached approximately  $10 \ \text{mm}^3$ , treatments with 20 IFN $\alpha$ /PVP or EP/PVP started and were repeated at 1-2 day intervals for 2 weeks (total of 8 treatments: Tumor volume was measured with electronic caliper in the two perpendicular diameters and in the depth. Measurements of the tumor masses  $(mm^3)$  were performed twice a week for 40-5025 days. All mice bearing tumor masses exceeding 1 cm3 volume were sacrificed for humane reasons. When depletion immunocompetent cells in vivo was required, a group of mice received i.v 0.5 ml of  $\alpha$ -CD4 (GK1.5 hybridoma, 207-TIB, 30 ATCC, Rockville, MD) ascite (1:10), or α-CD8 210-TIB, ATCC) ascite (1:100) or i.p. 100  $\mu g$ hybridoma, (RB6-8C5 hybridoma, Pharmingen, San Diego, CA). Control mice received i.v. 0.5 ml isotype control (Pharmingen). Antibody treatments were performed twice: first injection 1 day before starting the gene therapy 35

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treatment and the second injection (i.p at the same dosage) 7 days later.

#### CTL assay

A standard 6-hour 51-chromium (51Cr)-release assay was performed following 5 days of in vitro effector cell stimulation. Single cell suspensions of splenocytes were prepared 3 weeks following tumor challenge by mashing the spleens in RPMI 1640 medium (Life Technologies) and passing the cells through 70  $\mu m$  nylon mesh cell strainers (Falcon, 10 Becton Dickinson, Lincoln Park, NJ) into 50ml centrifuge tubes (Falcon). After centrifugation, red blood cells were lysed with ACK Lysing Buffer (Biofluids, Inc., Rockville, MD) and the splenocytes washed twice with RPMI. stimulation cultures contained 3 X 106 splenocytes/effectors 15 per ml with 6 X 10<sup>5</sup> mitomycin-C-treated Renca/stimulator cells per ml and 10 Units per ml recombinant murine IL-2 (Genzyme, Cambridge, MA) in RPMI containing 10% FBS, 22mM HEPES buffer (Research Organics Inc., Cleveland OH), Penn-5  $10^{-5}$ Streptomycin, Х M  $2-\beta$ -mercapto-ethanol 20 Technologies), OPI media supplement (Sigma, St. Louis, MO), essential and non-essential amino acids Technologies) (for a 5 : 1 responder : stimulator ratio). Stimulators were prepared by incubating Renca cells at 3 X  $10^7$  per ml in RPMI with 30  $\mu g$  per ml mitomycin-C (Sigma) at  $37^{\circ}$  C for 60 minutes, followed by four washes in HBSS with 25 2.5% FBS. After 5 days at 37° C, effector cells were pelleted, resuspended in complete RPMI, counted, and mixed with 51Cr -labeled targets in a 96 well V-bottomed plate (Costar/Corning, Cambridge, MA). Renca and CT26 targets 30 were labeled by incubating them at 2 X 10<sup>6</sup> cells per ml in complete RPMI with 150 uCi 51Cr (Amersham) for 2.5 hours. Targets were washed 3 times in HBSS with 2.5% FBS and resuspended in complete RPMI before addition to the assay. After mixing effectors and targets (in triplicate wells) and a brief pelleting, plates were placed at 37° C for 6 hours. 35

Approximately 90% of the supernatants were then collected from each well with the Skatron Harvesting Press Supernatant Collection System (Skatron Instruments, Norway). release was detected using a WALLAC 1470 automatic gamma counter (WALLAC Inc., Gaithersburg MD). Specific release was determined with the following equation: (experimental cpm spontaneous cpm) / (total spontaneous X 100. cpm) Spontaneous release from the targets was less than 18%, and the standard error of the triplicate experimental counts was less than 14%.

#### Statistical analysis

Data for the effects of mIFN- $\alpha$  gene therapy on tumor growth were analyzed by repeated measures analysis. Individual treatment means were compared using Duncan's multiple range test when the main effect was significant. for the effect of mIFN- $\alpha$  gene therapy on tumor rejection were analyzed by ANOVA. In all cases a p value of than 0.05 was considered to statistically be significant.

#### 20 Results

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## Expression of mIFN- $\alpha$

Murine IFN-α expression plasmid (pIF0836) transfected into Cos-1 cells, and the resulting conditioned media was assayed for mIFN- $\alpha$  by Western blot bioassay. Western blot analysis of conditioned media indicated that the recombinant mIFN-  $\alpha$  expressed from pIF0836 template was present as a single band with an approximate molecular weight of 23 kDa. This band was not observed in conditioned media from mock-transfected cells and likely represents a glycosylated form of mIFN- $\alpha$ . Recombinant mIFN- $\alpha$  ran with an approximate molecular weight of 18 kDa, which corresponds to the predicted molecular weight glycosylated mIFN- $\alpha$ . Assay of conditioned media using an

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anti-viral bioassay for mIFN- $\alpha$  indicated that approximately 175 x 10<sup>3</sup> IU/ml mIFN- $\alpha$  were present.

Anti-tumor activity of IFN- $\alpha$  gene therapy. tumor effect of murine  $IFN\alpha 4$  plasmid formulated as a complex with PVP (IFN $\alpha$ /PVP) was tested in a syngeneic murine renal cell carcinoma (Renca) and a mammary adenocarcinoma (TS/A) BALB/c mice were challenged subcutaneously tumor model. with 7  $\times 10^5$  Renca or 1  $\times 10^5$  CT26 cells, and IFN $\alpha$ /PVP injections were initiated 7 days later when tumors reached approximately 10 mm3 size. Each group of mice received at interval of 1-2 days 8 treatments (4 injections/week) of IFN $\alpha$ /PVP at scalar doses (from 12 to 96  $\mu$ g/mouse), EP/PVP (96  $\mu$ g/mouse) or no treatments for control (ctrl). size increased progressively in mice injected with EP/PVP (Renca, TS/A) or low doses of IFNα/PVP (TS/A), while tumors in mice injected with each dose of IFNa/PVP (Renca) or high dose of IFNa/PVP (TS/A) showed marked growth inhibition.

# Tumor growth inhibition is associated to systemic immune response

Treatments of Renca and TS/A tumors with IFN $\alpha$ /PVP at 96  $\mu$ g/mouse and 48  $\mu$ g/mouse respectively, induced complete regression in 6 out of 20 (Renca) and 2 out of 20 (TS/A) of challenged mice. To test whether the rejection of these tumors leads to specific immune memory, mice with no tumors for 40-50 days following IFN $\alpha$  treatments were re-challenged with a greater number of fresh tumors in the right flank. All mice that rejected primary tumors displayed protection against the second tumor challenge whereas mice used as the control group and injected for the first time with same number of tumor cells (1 X  $10^6$  Renca or 2 X  $10^5$  TS/A) developed tumors.

To evaluate the requirements for the induction of antitumor immune memory, Renca and TS/A were injected into BALB/c rendered immunosuppressed by treatment with anti-CD4, anti-CD8 or anti-polymorphonuclear cells (PMN). Depletion of CD8<sup>+</sup> T cells allowed both Renca and TS/A to grow in all animals following IFN $\alpha$ /PVP treatments, showing that this population is crucial for the immune response induced by IFN $\alpha$  gene therapy. No increase in tumor growth was found in mice treated with anti-PMN ( $\alpha$ -GR1) monoclonal Ab (mAb). Increase in tumor rejection was observed in mice depleted of CD4<sup>+</sup> T and treated with IFN $\alpha$ /PVP suggesting that depletion of CD4<sup>+</sup> T cells can enhance the anti-tumor effect of IFN $\alpha$  gene therapy.

Expression of IFN- $\alpha$  within the tumor induces a CTL 10 To assess whether CD8+ tumor specific CTL were induced in vivo by  $IFN\alpha/PVP$  treatments, splenocytes from Renca tumor-challenged mice were tested for their cytolytic activity following IFN $\alpha$  gene therapy. Cytotoxic activity against Renca, and not against CT26 cells used as control 15 for tumor specificity, was found in 2 of 4 mice that had received IFN $\alpha$  gene therapy. Moreover, splenocytes from mice depleted T cells and treated with of CD4<sup>+</sup> IFNa/PVP demonstrated potent CTL activity against Renca cells. 20 little CTL activity against Renca cells was contrast, evident from splenocytes isolated from mice treated with EP/PVP.

#### Discussion

The data reported herein demonstrate that direct 25 administration of IFN $\alpha$  gene formulated in a polymeric delivery system into subcutaneous renal cell carcinoma and mammary adenocarcinoma inhibits tumor growth and induces a long-lasting immunity to secondary tumor challenges. considerable significance is the fact that the anti-tumor 30 response was observed against both an immunogenic carcinoma well а more aggressive and poorly immunogenic adenocarcinoma, a phenotype which is similar spontaneously arising tumors in man.

A variety of genetic abnormalities arise in human 35 cancers that contribute to neoplastic transformation and

PCT/US99/05394 WO 99/47678

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Despite increasing understanding of the malignancy. malignancies many cancer, basis of molecular resistant to established forms of treatment. More recently, molecular genetic interventions have been designed in an attempt to improve the efficacy of immunotherapy. numerous experimental studies have been performed in murine models with tumor cells transduced with cytokine-gene ex vivo, a major limitation in the translation of this strategy to large-scale human tumor vaccine therapy is the labor intensity and variability of establishing each individual tumor in culture and transducing it with an appropriate Our approach to this problem is vector (i.e., retrovirus). to use a non-viral delivery system to modify tumor cells in vivo with cytokine cDNAs so that the tumor cells can supply the cytokine of interest in a paracrine fashion to the anti-15 tumor responder cells present within the tumor.

Using a plasmid containing IFNlpha4 gene and formulated in PVP, we have shown that intra-tumoral injections of this DNA-PINC complex can lead to complete tumor regression in 30 % of the cases (Renca model) with an overall response rate 20 These results are in tumor growth inhibition. of 100 % agreement with a recent study that described an anti-tumor TS/A genetically modified by activity elicited producing murine IFN- $\alpha$ 1 (Ferrantini et al., 1994). Although the anti-tumor effect of IFN $\alpha$  using cytokine-gene transduced 25 al., described (Scarpa cells been has tumor Murine Mamary in а Remodelling Matrix "Extracellular Adenocarcicoma Transfected with the Interferon-alphal Gene", Journal of Pathology. 181, 116-123 1997), the real value of IFN $\alpha$  gene therapy in blocking or inhibiting advanced tumors 30 The advantage of using a non-viral remains to be explored. IFN $\alpha$  gene delivery system over retrovirus is that tumor cells could be transduced directly in vivo without the need of first establishing tumor cells in vitro. Moreover, IFNa has a potent anti-viral activity limiting the use of this 35 gene in combination with viral vectors.

Therapeutic levels of gene expression for IGF-I using a similar interactive PVP-based delivery system have previusly been described (Alila et al., 1997). Direct intra-tumor injection of the same PINC delivery system as a complex with IFN $\alpha$  gene, resulted in dispersion in vivo of plasmid into target cells inducing an IFN $\alpha$ -specific anti-tumor activity. Tumors treated with the same plasmid but in the absence of IFN $\alpha$  coding region and formulated as a complex with PVP, did not respond to this treatment and grew in a similar rate to untreated tumors. By using an optimal dose of IFN $\alpha$ /PVP, tumor-bearing mice were able to reject the tumors mounting a specific long-lasting tumor immunity. Although, the numbers of mice rejecting a second tumor challenge was low, this observation indicates that a considerable portion of the activity of IFN $\alpha$  in inducing the rejection of established tumors is not anti-angiogenic or anti-proliferative immunostimulatory. Our result demonstrating induced regression of advanced tumors was prevented by in vivo administration of anti-CD8 mAb provides direct evidence for a key role of  $CD8^+$  T cells in the anti-tumor effect of

Depletion of  $CD4^+$  T cells in tumor-bearing mice treated with gene therapy significantly enhanced therapeutic effect of IFN $\alpha$ , resulting in tumor regression and prolonged survival of up to 80% of treated mice. A CD4-25 mediated suppression during tumor progression previously has reported and it has also been shown depletion of  $CD4^+$  T cells in tumor-bearing mice results in augmentation of anti-tumor therapy with either IL-2 or IL-12 (Rackmilevich et al., 1994 and Martinotti et al., "Cd4 T 30 Cells Inhibit in Vivo the Cd8-Mediated Immune Response Murine Colon Carcinoma Cells Transducted Interleukin-12 Genes", Eur. J. Immunol, 25, (1995)). They have shown that depletion of CD4  $^{+}\mathrm{T}$  cells in tumor-bearing mice in the absence of treatment did not 35 affect the growth of tumor itself suggesting that removal of

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 $\mathtt{CD4}^+$  T cells does not deprive the tumor of growth factors (Rackmilevich et al., 1994). A possible explanation for this phenomenon is that depletion of CD4 T cells from tumorbearing mice augments the anti-tumor efficacy of IFNαreleasing them cells by CD8<sup>+</sup> Т activated immunosuppression. The mechanism driving CD4 suppression is poorly understood, although Th2 type cytokines, directly or inhibit cell-mediated cell activation, may through В immunity (Mossman et al., 1989; Powrie et al., Eur-J-CTL can be generated in Immunol, 23(11):3043-9 (1993)). 10 both CD4-depleted and non-depleted mice from lymphocytes obtained from spleens by in vitro re-stimulation with mitomycin-treated Renca cells and rIL-2. Thus, CD4-mediated suppression appears to be exerted on CD8 expansion and not In accord with the in vivo results, stronger CTL priming. 15 activity was observed from mice depleted of CD4 and treated with IFNlpha/PVP suggesting CD4 $^+$  T cells inhibit an IFNlphamediated CD8 T cell response in vivo. This study suggests that direct administration of cytokine genes, like IFN $\alpha$ , into tumors, which have been found to suppress malignancy growth, provide a new therapeutic option for the treatment of human cancers.

# Example 2: Pharmacology of mIFN - Gene Medicine in Syngeneic Tumor Models

Gene expression systems encoding either mIFN-2 or mIFN-4 were tested for anti tumor activity when formulated in either cationic lipid, peptide, or PINC delivery systems and injected intratumorally into subcutaneous squamous cell carcinoma (SCC-VII) or adenocarcinoma (MC-38) tumors.

## 30 Experimental design and treatment regimen

Experiments to test the anti tumor activity of mIFN-gene medicine were conducted in either SCC-VII or MC-38 tumor models. Tumor cells (4 x  $10^5$ ) were injected subcutaneously into the flank region of mice, and treatment

was initiated when tumor volume reached approximately 50 mm<sup>3</sup>. Treatment was begun approximately 6 days (SCC-VII tumors) and 10 days (MC-38 tumors) after tumor initiation and repeated at 3 to 5 day intervals.

All formulations of mIFN- gene medicine were administered in a dose volume of 50 μl. The faster growing SCC-VII tumors typically received 3 treatments, whereas the relatively slower growing MC-38 tumors typically received 4 treatments. Experiments were terminated when lactose vehicle control tumors reached approximately 1000 mm<sup>3</sup>.

The anti-tumor effects of murine IFN gene medicine (IFNa/PVP) was tested in syngeneic murine carcinoma (Renca) and mammary adenocarcinoma (TS/A) tumor renal BALB/c mice were challenged subcutaneously with 7  $X10^5$  or 1  $X10^5$  CT26, and IFN  $\alpha/\text{PVP}$  injections were initiated 7 15 days later when tumors reached approximately 10 mm3 size. Each group of mice received 8 treatments (4 injections for 2 weeks) of IFN $\alpha$ /PVP at scalar doses (from 12 to 96  $\mu$ g/mouse), empty plasmid/PVP (EP/PVP, 96  $\mu$ g/mouse) or no treatments for 20 control (ctrl). Tumor size increased progressively in mice injected with EP/PVP (Renca, TS/A) or low doses of IFN $\alpha$ /PVP (TS/A), while tumors in mice injected with each dose of IFN $\alpha$ /PVP (Renca) or high dose of IFN $\alpha$ /PVP (TS/A) marked growth inhibition.

# 25 Example 3: mIFN- Gene Medicine Formulated in Cationic lipid Reduces the Growth of SCC-VII Tumors

Experiments were conducted in the SCC-VII tumor model as described in the preceding example. mIFN- gene medicine formulated in cationic lipid, peptide, and PINC delivery 30 systems was tested. Results show that cationic lipid formulations significantly reduce the growth of SCC-VII tumors relative both to lactose vehicle injected tumors and tumors injected with control (non coding) formulated in cationic lipid. plasmid The effect of mIFNmedicine formulated in cationic lipid is dose dependent and 35

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there is no effect of mIFN- gene medicine when formulated in PVA. In addition, analysis of tumors from this experiment using immunohistochemical methods revealed infiltration of CD8+ lymphocytes in tumors injected with cationic lipid formulations, but not in tumors injected with PVA formulations.

mIFN- gene medicine significantly reduces the growth of SCC-VII tumors as compared to control plasmid or lactose injected tumors. Differences between control plasmid and mIFN- plasmid were consistent across formulation. Plasmid dose was 46  $\mu$ g/treatment. Growth of tumors injected with control plasmid was compared to that of tumors injected with mIFN- gene medicine using repeated measures analysis. mIFN- reduced SCC-VII tumor growth relative to control plasmid (p=.035).

# Example 4: mIFN- Gene Medicine Reduces the Growth of MC-38 Tumors

Experiments were carried out as described in the preceding examples. The effects of various prototype formulations of mIFN- gene medicine on the growth of subcutaneous MC-38 tumors were compared. mIFN- gene medicine elicited reduced tumor growth in all formulations tested (cationic lipid, peptide, and PINC). Subsequent experiments in the MC-38 tumor model have shown similar results.

#### Example 5: Dose Responses

After demonstrating anti tumor effects of mIFN- gene medicine, the dose response for these effects was investigated in the MC-38 tumor model. Both cationic lipid (DOTMA:Chol) and PINC (PVA) delivery systems were evaluated. Results clearly show that mIFN- gene medicine elicited a dose dependent reduction in tumor growth. Tumor volume in this experiment was maximally reduced by approximately 50 % with mIFN- /DOTMA:Chol and 60 % with mIFN- /PVA after 4

treatments. Maximal reduction in tumor volume was observed at plasmid dose of approximately 50 µg/treatment (cumulative dose approximately of 200 μq). These experiments will be conducted primarily in the MC-38 tumor model because it provides a broader treatment window than does the SCC-VII model.

## Example 6: IFN-alpha Formulations

The formulations for the IFN-a are: (1) PVP 4 vial, (2) PVP three vial, (3) PVP two vial. The details are listed below:

#### PVP 4 vial

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Materials: 25% PVP (50 kDa) stock solution, plasmid stock solution, 5 M NaCl stock solution, and water.

Method: Add in order of water, plasmid, 25% PVP and 5 M NaCl into a vial to make a plasmid in 5% PVP in saline formulation. The final concentration of PVP and NaCl are fixed (5% and 150 mM) and plasmid concentration could be varied (but based on the IGF-1 data, 3 mg DNA/ml in 5% PVP in saline should be the best formulation). The quality of the formulation is characterized by pH, DNA concentration, osmolality, and gel electrophoresis. The DNA concentration could be varied from 0.1-5 mg/ml. The pH may be varied from 3-5, osmolality may be 250 - 400 mOsm.

#### Three vial

Material: lyophilized PVP, plasmid stock solution (4 mg/ml), 115 mM Na-Citrate/5% NaCl stock buffer (pH = 4).

Method: Add in order of plasmid and buffer into the PVP to make final 3 mg DNA/ml in 5% PVP in 25 mM citrate/saline buffer (pH =4). DNA expression is higher in saline than in the citrate buffer.

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#### Two vial

Materials: Co-lyophilized plasmid and PVP, saline. Add saline into the co-lyophilized DNA and PVP to make final 3 mg/ml DNA in 5% PVP in saline.

The final formulation is 3mg/mL DNA, 5% PVP as a single vial. The formulation is prepared by adding (5%) PVP to (4mg/mL) DNA and recirculating the two components for a finite period of time (using static mixer). Then the formulation is lyophilized and rehydrated with 0.9% sodium chloride, to obtain a final composition of 3mg/mL, 5%PVP in saline.

# Example 7: Treatment of Human Tumors

Human tumors to therapy using a plasmid construct encoding the human IFN alpha gene sequence such as that depicted in SEQ ID NO: 10, 11 or 12. A patient in need of anti-cancer therapy is injected with up to 3mg of plasmid formulation at daily intervals. The plasmid formulation may contain INF alpha plasmid alone or optionally a mixture of IFN-alpha encoding plasmid and an additional plasmid coding for a cytokine. The preffered cytokine is IL-12. The treatments are continued and the patient monitored as is the usual practice for anti-cancer chemotherapeutic regimes.

One skilled in the art would readily appreciate that 25 the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The molecular complexes and the methods, procedures, treatments, molecules, 30 compounds described herein are presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims. 35

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It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. For example, if X is

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described as selected from the group consisting of bromine, chlorine, and iodine, claims for X being bromine and claims for X being bromine and chlorine are fully described.

Other embodiments are within the following claims.

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#### Claims

- 1. A plasmid comprising a CMV promoter transcriptionally linked with an interferon alpha coding sequence, and a growth hormone 3'-untranslated region.
- 5 2. The plasmid of claim 1, wherein said interferon alpha is human interferon alpha.
  - 3. The plasmid of claim 2, wherein said human interferon alpha coding sequence is a synthetic sequence having optimal codon usage.
- 4. The plasmid of claim 3, wherein said interferon alpha coding sequence has the nucleotide sequence of SEQ ID NO:10, 11 or 12.
- The plasmid of claim 1, wherein said growth hormone 3' untranslated region is from a human growth
   hormone gene.
  - 6. The plasmid of claim 5, wherein an ALU repeat or ALU repeat-like sequence is deleted from said 3' untranslated region.
- 7. The plasmid of claim 1, wherein said plasmid includes a promoter, a TATA box, a Cap site and a first intron and intron/exon boundary in appropriate relationship for expression of said coding sequence.
- The plasmid of claim 7, wherein said plasmid further comprises a 5' mRNA leader sequence inserted between
   said promoter and said coding sequence.

- 9. The plasmid of claim 1, wherein said plasmid further comprises an intron/5' UTR from a chicken skeletal  $\alpha$ -actin gene.
- 10. The plasmid of claim 1, wherein said plasmid 5 comprises a nucleotide sequence which is the same as the nucleotide sequence of plasmid pIF0921.
  - 11. The plasmid of claim 1, further comprising:
  - a first transcription unit comprising a first transcriptional control sequence transcriptionally linked with a first 5'-untranslated region, a first intron, a first coding sequence, and a first 3'-untranslated region/poly(A) signal, wherein said first intron is between said control sequence and said first coding sequence; and
- a second transcription unit comprising a second transcriptional control sequence transcriptionally linked with a second 5'-untranslated region, a second intron, a second coding sequence, and a second 3'-untranslated region/poly(A) signal, wherein said second intron is between said control sequence and said second coding sequence;
- wherein said first and second coding sequences comprise a sequence having the sequence of SEQ ID NO:2, 3, 4 or 25 coding for human IL-12 p40 subunit, and a sequence having the sequence of SEQ ID NO:6, 7, 8 or 24 coding for human IL-12 p35 subunit.
- 25 12. The plasmid of claim 11, wherein said first transcriptional control sequence or said second transcriptional control sequence comprise one or more cytomegalovirus promoter sequences.
- 13. The plasmid of claim 11, wherein said first and 30 second transcriptional control sequences are the same.

- The plasmid of claim 11, wherein said first and second transcriptional control sequences are different.
- The plasmid of claim 14, wherein said sequence coding for the p40 subunit of human IL-12 is 5' to said sequence coding for the p35 subunit of human IL-12.
  - The plasmid of claim 1, further comprising an intron having variable splicing, a first coding sequence, and a second coding sequence,
- wherein said first and second coding sequences comprise a sequence having the sequence of SEQ ID NO:2, 3, 410 or 25 coding for human IL-12 p40 subunit, and a sequence having the sequence of SEQ ID NO:6, 7, 8 or 24 coding for human IL-12 p35 subunit.
  - The plasmid of claim 16, further comprising:
- 15 transcriptional control transcriptionally linked with a first coding sequence and a second coding sequence;
  - a 5'-untranslated region;
  - an intron 5' to said first coding sequence;
- 20 an alternative splice site 3' to said first coding sequence and 5' to said second coding sequence; and a 3'-untranslated region/poly(A) signal.
- The plasmid of claim 17. wherein transcriptional control sequence comprises a cytomegalovirus 25 promoter sequence.
  - The plasmid of claim 1, further comprising:
- а transcriptional control transcriptionally linked with a first coding sequence, an sequence, second coding sequence, а and untranslated region/poly(A) 30 signal, wherein said

sequence is between said first coding sequence and said second coding sequence; and

an intron between said promoter and said first coding sequence;

- wherein said first and second coding sequences comprise a sequence having the sequence of SEQ ID NO:2, 3, 4 or 25 coding for human IL-12 p40 subunit, and a sequence having the sequence of SEQ ID NO:6, 7, 8 or 24 coding for human IL-12 p35 subunit.
- 10 20. The plasmid of claim 19, wherein said transcriptional control sequence comprises a cytomegalovirus promoter sequence.
  - 21. The plasmid of claim 19, wherein said IRES sequence is from an encephalomyocarditis virus.
- 22. A composition comprising the plasmid of anyone of claims 1-21, and a protective, interactive non-condensing compound.
- 23. The composition of claim 22, wherein said protective, interactive non-condensing compound is polyvinyl 20 pyrrolidone.
  - 24. The composition of claim 22, wherein said plasmid is in a solution having between 0.5% and 50% PVP.
  - 25. The composition of claim 24, wherein said solution includes about 5% PVP.
- 25 26. The composition of claim 22, wherein said DNA is at least about 80% supercoiled.
  - 27. The composition of claim 26, wherein said DNA is at least about 90% supercoiled.

- 28. The composition of claim 27, wherein said DNA is at least about 95% supercoiled.
- 29. A composition comprising a protective, interactive non-condensing compound and a plasmid comprising an interferon alpha coding sequence.
  - 30. A composition comprising the plasmid of any one of claims 1-21 and a cationic lipid with a neutral colipid.
- 31. The composition of claim 30, wherein said cationic lipid is DOTMA.
  - 32. The composition of claim 30, wherein said neutral co-lipid is cholesterol.
- 33. The composition of claim 30, wherein the DNA in said plasmid and said cationic lipid are present in such amounts that the negative to positive charge ratio is about 1:3.
  - 34. The composition of claim 30, wherein said DNA is at least about 80% supercoiled.
- 35. The composition of claim 34, wherein said DNA is 20 at least about 90% supercoiled.
  - 36. The composition of claim 35, wherein said DNA is at least about 95% supercoiled.
  - 37. The composition of claim 30, further comprising an isotonic carbohydrate solution.

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- 38. The composition of claim 37, wherein said isotonic carbohydrate solution consists essentially of about 10% lactose.
- 39. The composition of claim 30 wherein said cationic lipid and said neutral co-lipid are prepared as a liposome having an extrusion size of about 800 nanometers.

#### 40. A composition comprising:

- a first component comprising a plasmid comprising an interferon alpha coding sequence and a cationic lipid with a neutral co-lipid, wherein said cationic lipid is DOTMA and said neutral co-lipid is cholesterol, wherein the DNA in said plasmid and said cationic lipid are present in amounts such that the negative to positive charge ratio is about 1:3; and
- a second component comprising a protective, interactive non-condensing compound, wherein said first component is present within the second component.
- 41. A composition comprising a protective, interactive non-condensing compound, a first plasmid comprising an interferon alpha coding sequence, and one or more other plasmids independently comprising an IL-12 or IL-12 subunit coding sequence.
- 42. A composition comprising a plasmid comprising an interferon alpha coding sequence and a cationic lipid with a 25 neutral co-lipid.
  - 43. A method for making a plasmid of anyone of claims 1-21 comprising the step of inserting a CMV promoter transcriptionally linked with an interferon alpha coding sequence, and a growth hormone 3'-untranslated region into a plasmid.

- 44. A method for making a composition of claim 29, comprising the steps of:
- a. preparing a DNA molecule comprising a transcriptional unit, wherein said transcriptional unit comprises an interferon alpha coding sequence;
- b. preparing a protective, interactive noncondensing compound; and
- c. combining said protective, interactive non-condensing compound with said DNA in conditions such that a composition capable of delivering a therapeutically effective amount of an interferon alpha coding sequence to a mammal is formed.
- 45. The method of claim 44 wherein said DNA molecule is a plasmid, wherein said plasmid comprises a CMV promoter transcriptionally linked with an interferon alpha coding sequence, and a human growth hormone 3'-untranslated region/poly(A) signal.
  - 46. A method of making a composition of claim 30, comprising the steps of:
- a. preparing a DNA comprising an interferon alpha coding sequence;
  - b. preparing a mixture of a cationic lipid and a neutral co-lipid, wherein said cationic lipid is DOTMA and said neutral co-lipid is cholesterol; and
- C. combining said mixture with said DNA in amounts such that said cationic lipid and said DNA are present in a negative to positive charge ratio of about 1:3.
  - $47.\ \ A$  method of making a composition of claim 40, comprising the steps of:
- a. preparing a first component comprising a plasmid comprising an interferon alpha coding sequence and a cationic lipid with a neutral co-lipid, wherein said cationic lipid is DOTMA and said neutral co-lipid is

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cholesterol, wherein the DNA in said plasmid and said cationic lipid are present in amounts such that the negative to positive charge ratio is about 1:3;

- b. preparing a second component comprising a protective, interactive non-condensing compound; and
  - c. combining said first and second components such that the resulting composition comprises said first component within said second component.
- 48. A method of making a composition of claim 41, 10 comprising the steps of:
  - a. preparing a protective, interactive noncondensing compound,
  - b. preparing a first plasmid comprising an interferon alpha coding sequence,
- 15 c. preparing one or more other plasmids independently comprising an IL-12 p35 or IL-12 p40 subunit coding sequence, and
- d. combining said protective, interactive noncondensing compound, said plasmid comprising said interferon
   20 alpha coding sequence and said other plasmids.
  - 49. A method of making a composition of claim 42 comprising combing a plasmid comprising a interferonalpha coding sequence and a cationic lipid with a neutral colipid.
- 50. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a plasmid of anyone of claims 1-21.
- 51. The method of claim 50, wherein said condition or 30 disease is a cancer.

- 52. The method of claim 50, wherein said composition is administered by injection.
- 53. A method for transfection of a cell *in situ*, comprising the step of contacting said cell with a plasmid of anyone of claims 1-21 for sufficient time to transfect said cell.
  - 54. The method of claim 53, wherein transfection of said cell is performed in vivo.
- 55. The method of claim 53, wherein said contacting is performed in the presence of an about 5% PVP solution.
  - 56. A method for delivery and expression of an interferon alpha gene in a plurality of cells, comprising the steps of:
- (a) transfecting said plurality of cells with a 15 plasmid of anyone of claims 1-21; and
  - (b) incubating said plurality of cells under conditions allowing expression of a nucleic acid sequence in said vector, wherein said nucleic acid sequence encodes interferon alpha.
- 57. The method of claim 56, wherein said interferon alpha is human interferon alpha and said cells are human cells.
  - 58. The method of claim 56, wherein said contacting is performed in the presence of an about 5% PVP solution.
- 25 59. A method for treating a disease or condition, comprising the steps of transfecting a cell *in situ* with a plasmid of any one of claims 1-21.

- 60. The method of claim 59, wherein said disease or condition is a localized disease or condition.
- 61. The method of claim 59, wherein said disease of condition is a systemic disease or condition.
- 5 62. A cell transfected with a plasmid of anyone of claims 1-21.
- 63. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of claim 22.
  - 64. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of claim 29.
- 65. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of claim 30.
- 66. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of claim 40.
- 67. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of claim 41.
  - 68. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from

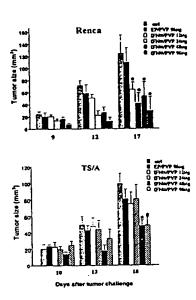
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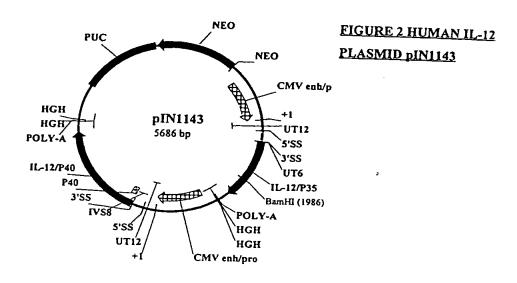
said condition or disease a therapeutically effective amount of a composition of claim 42.

69. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of a first plasmid comprising an interferon alpha coding sequence and a second plasmid comprising a IL-12 coding sequence.

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### FIGURE 1





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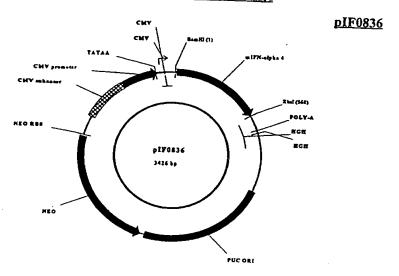
### FIGURE 3

### Codon Frequency

### human\_high.doc

Codon usage Lo	r GCC an	(Fighly expre	ssedi genes	1631/91
УБУ	<b>664</b> on	<b>№6</b> ££6€	/1000s Fr	rc Ojtan
Gly	GGT	441.00	9.14	0.12
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Am	GTT	198.00	4.10	0.01
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Lys	AAG	2117.00	43.88	0.82
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## FIGURE 4 MOUSE INTERFERON ALPHA

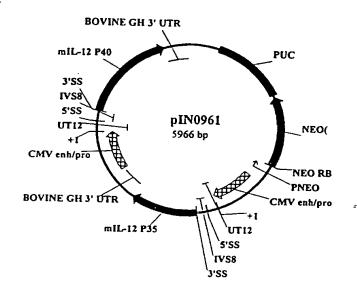


gate categoriag get at get at gate categoriag at gate at gategoriag gate at gate at gategoriag gate at gategoriag gategat a accase caga agget case centering the constraint of the consctctcttcctcaaccaacttgctggcaagactgagtgaggagaaggagtgatctagaaagccgaattctgcaggaattttggcatccctgtgacccctcccca gtgcctctcctggccctggaagttgccactccagtgcccaccagccttgtcctaataaaattaagttgcatcattttgtctgactaggtgtccttctataatattatg gggtta atticg agett ggcgta at cat ggt cat aget gttte ct gt gt gaa att gtt at ceget caca attic caca caca cat ac gag ceg gaa gcat aa agt gt to get the control of the contra a a geot to geg g to consider a constraint to the constraint teggeeaaegegegggagaggeggtttgegtattgggegetetteegetteetegeteaetgaetegetgegeteggtegtteggetgeggegageggtat cagcit cact cas agg c g taat acg g t ta t cca cag a at cag g g g at a acg cag g a a a a g g c cag g a a acg g ta ta c g g a acc g ta ta can a g g c cag g a acc g ta cag g a acc g a acc g ta cag g a acc gtata a agata c cagge gette cecteg gaage te ceteg te gette ceget te ceget accege gata cet get e cette te cette en gette ce de la companyation degtggcgctttctcatagctcacgctgtaggtatctcagttcggtgtaggtcgttcgctccaagctgggctgtgtgcacgaacccccgttcagcccgaccgct gegeettateeggtaaetategtettgagteeaaeeeggtaagaeaegaettategeeaetggeageageaetggtaaeaggattageagagegaggtat gtaggeggtgctacagagttcttgaagtggtggcctaactacggctacactagaaggacagtatttggtatctgcgctctgctgaagccagttaccttcggaa

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#### FIGURE 5 MOUSE IL-12

### **pIN0961**



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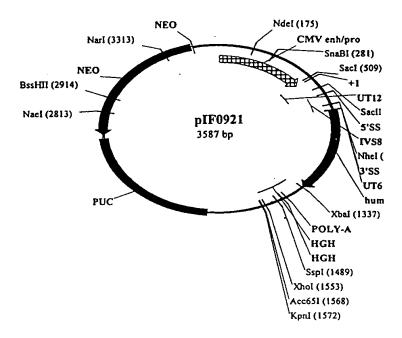
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### FIGURE 6 HUMAN IFN

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Figure 7A IFNplas

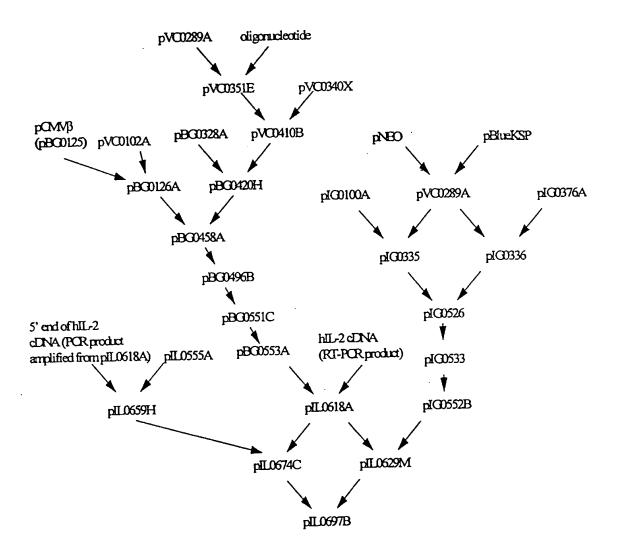


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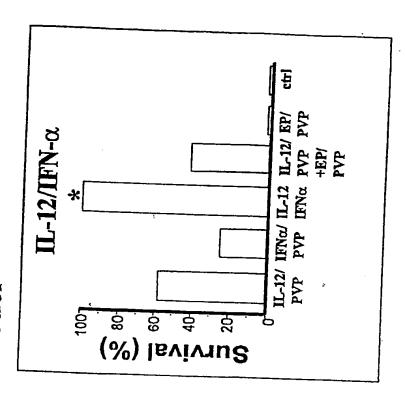
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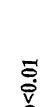
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### Figure 8



IL-12 Gene Medicine (Combination Therapy) in Renca Model FIGURE 9









IL-12/PVP (24  $\mu$ g) IL-2/DC (6  $\mu$ g) IFN- $\alpha$  (96  $\mu$ g) EP= empty plasmid/PVP (96  $\mu$ g)

### Sequence Listing Part

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	<120	١,						RO:			AND	DELI	VER	Y SY	STEM	s.
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# INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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US

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, Fl, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

#### Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

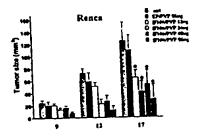
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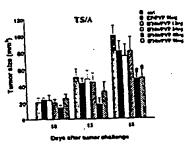
9 December 1999 (09.12.99)

(54) Title: INTERFERON ALPHA PLASMIDS AND DELIVERY SYSTEMS, AND METHODS OF MAKING AND USING THE SAME

#### (57) Abstract

The present invention relates to gene delivery and gene therapy, and provides novel nucleic acid constructs for expression of interferon alpha in a mammal, formulations for delivery that incorporate a nucleic acid construct for expression, and methods for preparing and using such constructs and formulations. In particular, this invention relates to plasmid constructs for delivery of therapeutic interferon alpha encoding nucleic acids to cells in order to modulate tumor activity, methods of using those constructs (including combination therapy with other agents, such as cytokines, preferably IL-12), as well as methods for preparing such constructs.





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## INTERNATIONAL SEARCH REPORT

Pul/US 99/05394

L CLASSIFI IPC 6	CATION OF SUBJECT C12N15/21 C12N15/85	C07K14/56 A61K48/00	C12N15/88 C12N5/10	C12N15/24 //A61K9/127	C07K14/54	
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- DOCUME	ENTS CONSIDERED 1	O BE RELEVANT				
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	6 June 19	96 (1996-06-0	16)		33-36,	
					42-45,	
					49-66,68	
	the whole	the whole document especially page 46, line 1 - line 37; claims; figure				
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X F	uther documents are li	sted in the continuation of	of box C.	X Patent family memo	gers allo sasto in English	
' Special	categories of cited doc	uments		T" later document published	after the international filing date in conflict with the application but	
-AT docu	ment defining the gene	eral state of the art which	is not	cited to understand the invention	principle or theory underlying the	
l con	sidered to be of particu	plar relevance hed on or after the lintern		X* document of particular re	elevance; the claimed invention novel or cannot be considered to	
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"O" docu	ument referning to an o er means	ral disclosure, use, exhib		ments, such combinati	on being obvious to a person skilled	
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	NL - 2280 H Tel (+31-70) Fax: (+31-70	, 340-2040. Tx, 31 651 ep	oo ni.	Le Cornec	, N	

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	continuation of second sheet) (July 1992)	

ternational application No.

PCT/US 99/05394

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)
This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:
1. X Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely: Remark: Although claims 50-55, 59-61, 63-69 are directed to a method of treatment of the human/animal body, (rule 39.1 (IV) PCT, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).
Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)
This International Searching Authority found multiple inventions in this international application, as follows:
As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Remark on Protest  The additional search fees were accompanied by the applicant's protest.  No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

Information on patent family members

International Application No Pur/US 99/05394

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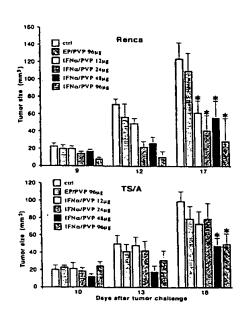
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#### (57) Abstract

The present invention relates to gene delivery and gene therapy, and provides novel nucleic acid constructs for expression of interferon alpha in a mammal, formulations for delivery that incorporate a nucleic acid construct for expression, and methods for preparing and using such constructs and formulations. In particular, this invention relates to plasmid constructs for delivery of therapeutic interferon alpha encoding nucleic acids to cells in order to modulate tumor activity, methods of using those constructs (including combination therapy with other agents, such as cytokines, preferably IL-12), as well as methods for preparing such constructs.



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#### DESCRIPTION

# Interferon Alpha Plasmids And Delivery Systems, And Methods Of Making And Using The Same

#### Related Applications

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This application relates to U.S. patent application Serial No. 08/949,160, filed October 10, 1997 and International patent application No. PCT/US97/18779, filed October 10, 1997, (Lyon & Lyon Docket Nos. 226/285 US and PCT, respectively), both of which are related to U.S. patent application Serial No. 60/028,676, filed October 18, 1996, (Lyon & Lyon Docket No. 222/086 US), all three of which are entitled "IL-12 GENE EXPRESSION AND DELIVERY SYSTEMS AND USES" (by Nordstrom et al.).

This application is also related to U.S. patent application Serial No. 08/798,974, filed February 11, 1997, (Lyon & Lyon Docket No. 224/084 US) and International patent application No. PCT/US95/17038, filed December 28, 1995, (Lyon & Lyon Docket No. 210/190 PCT), both of which are related to U.S. patent application Serial No. 08/372,213, filed January 13, 1995, (Lyon & Lyon Docket No. 210/190 US), all three of which are entitled "FORMULATED NUCLEIC ACID COMPOSITIONS AND METHODS OF ADMINISTERING THE SAME FOR GENE THERAPY" (by Mumper Rolland).

Each of the above-mentioned applications are 25 incorporated herein by reference in their entirety, including any drawings.

#### Field Of The Invention

The present invention relates to gene delivery and gene therapy, and provides novel nucleic acid constructs for expression of interferon alpha in a mammal, formulations for delivery that incorporate a nucleic acid construct for expression, and methods for preparing and using such constructs and formulations. In particular, this invention

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relates to plasmid constructs for delivery of therapeutic interferon alpha encoding nucleic acids to cells in order to modulate tumor activity, methods of using those constructs (including combination therapy with other agents, such as cytokines, preferably IL-12), as well as methods for preparing such constructs.

#### Background Of The Invention

The following discussion of the background of the invention is merely provided to aid the reader in understanding the invention and is not admitted to describe or constitute prior art to the present invention.

Plasmids are an important element in genetic engineering and gene therapy. Plasmids are usually circular DNA molecules that can be introduced into bacterial cells by transformation which replicate autonomously in the cell. Plasmids typically allow for the amplification of cloned DNA. Some plasmids are present in 20 to 50 copies during cell growth, and after the arrest of protein synthesis, as many as 1000 copies per cell of a plasmid can be generated. Suzuki et al., Genetic Analysis, p. 404, 1989.

Current non-viral approaches to human gene therapy require that a potential therapeutic gene be cloned into plasmids. Large quantities of a bacterial host harboring the plasmid may be fermented and the plasmid DNA may be purified for subsequent use. Current human clinical trials plasmids utilize this approach. Recombinant Advisory Committee Data Management Report, December, 1994, Human Gene Therapy 6:535-548. Studies normally focus on the therapeutic gene elements that and the control expression in the patient when designing and constructing gene therapy plasmids. Generally, therapeutic genes regulatory elements are simply inserted into existing cloning vectors that are convenient and readily available.

Plasmid design and construction utilizes several key 35 factors. First, plasmid replication origins determine

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plasmid copy number, which affects production yields. Plasmids that replicate to higher copy number can increase plasmid yield from a given volume of culture, but excessive copy number can be deleterious to the bacteria and lead to undesirable effects (Fitzwater, et al., Embo J. 7:3289-3297 (1988); Uhlin, et al., Mol. Gen. Genet. 165:167-179 (1979)). Artificially constructed plasmids are sometimes unstably maintained, leading to accumulation of plasmid-free cells and reduced production yields.

To overcome this problem of plasmid-free cells, genes 10 that code for antibiotic resistance phenotype are included on the plasmid and antibiotics are added to kill or inhibit plasmid-free cells. Most general purpose cloning vectors contain ampicillin resistance ( $\beta$ -lactamase, or bla) genes. ampicillin can be problematic because of 15 potential for residual antibiotic in the purified DNA, which can cause an allergic reaction in a treated patient. addition,  $\beta$ -lactam antibiotics are clinically important for When plasmids containing antibiotic disease treatment. resistance genes are used, the potential exists for the 20 transfer of antibiotic resistance genes to a potential pathogen.

Other studies have used the neo gene which is derived The neo gene encodes from the bacterial transposon Tn5. (Smith, and neomycin kanamycin resistance to This gene has been used in a number 12:1515-1519 (1994)). of gene therapy studies, including several human clinical trials (Recombinant DNA Advisory Committee Data Management Report, December, 1994, Human Gene Therapy 6:535-548). to the mechanism by which resistance is imparted, residual the gene to potential antibiotic and transmission of pathogens may be less of a problem than for  $\beta$ -lactams.

In addition to elements that affect the behavior of the plasmid within the host bacteria, such as  $E.\ coli$ , plasmid vectors have also been shown to affect transfection and expression in eukaryotic cells. Certain plasmid sequences

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have been shown to reduce expression of eukaryotic genes in eukaryotic cells when carried in cis (Peterson, et al., Mol. Cell. Biol. 7:1563-1567 (1987); Yoder et al., Mol. Cell. Biol. 3:956-959 (1983); Lusky et al., Nature 293:79-81 (1981); and Leite, et al., Gene 82:351-356 (1989)). Plasmid sequences also have been shown to fortuitously contain binding sites for transcriptional control proteins (Ghersa, et al., Gene 151:331-332 (1994); Tully et al., Biochem. Biophys. Res. Comm. 144:1-10 (1987); and Kushner, et al., Mol. Endocrinol. 8:405-407 (1994)). This can cause inappropriate levels of gene expression in treated patients.

Interferon alpha is a gene product that has been proposed for use, either alone or in combination with other agents, in different delivery systems for the treatment of 15 certain diseases, including particular cancers. International patent publication WO/97/00085, published January 3, 1997, proposes ex vivo transfection of tumor cells with interferon alpha and another immomodulatory molecule, such as IL-12. None of the previously proposed 20 treatments have proven entirely satisfactory, due in part to the high cost and technical difficulty involved in ex vivo Thus there still remains a need in the art for approaches. improved plasmids encoding interferon alpha as well as improved treatment protocols and technologies.

#### 25 Summary

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The present invention relates to gene delivery and gene therapy, and provides novel nucleic acid constructs for expression of interferon alpha in a mammal, formulations for delivery that incorporate a nucleic acid construct for expression, and methods for preparing and using such constructs and formulations. In particular, this invention relates to plasmid constructs for delivery of therapeutic interferon alpha encoding nucleic acids to cells in order to modulate tumor activity, methods of using those constructs (including combination therapy with other agents, such as

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cytokines, preferably IL-12), as well as methods for preparing such constructs. The pharmaceutical acceptable, cost effective and highly efficient delivery system presented herein represents an unanticipated improvement over the art.

Thus, in a first aspect, the invention features a plasmid that contains a CMV promoter and optionally a synthetic 5' intron transcriptionally linked with an interferon alpha coding sequence, and a 3'-untranslated region (UTR). Preferably the 3' UTR is a 3' growth hormone UTR.

As used herein, the term "plasmid" refers to a construct made up of genetic material (i.e., nucleic acids). It includes genetic elements arranged such that an inserted coding sequence can be transcribed in eukaryotic cells. Also, while the plasmid may include a sequence from a viral nucleic acid, such viral sequence does not cause the incorporation of the plasmid into a viral particle, and the plasmid is therefore a non-viral vector. Preferably a plasmid is a closed circular DNA molecule.

"Cytomegalovirus promoter" refers to one or more sequences from a cytomegalovirus which are functional in eukaryotic cells as a transcriptional promoter and an upstream enhancer sequence. The enhancer sequence allows transcription to occur at a higher frequency from the associated promoter.

In this context, "transcriptionally linked" means that in a system suitable for transcription, transcription will initiate under the direction of the control sequence(s) and proceed through sequences which are transcriptionally linked with that control sequence(s). Preferably no mutation is created in the resulting transcript, which would alter the resulting translation product.

The term "coding region" or "coding sequence" refers to a nucleic acid sequence which encodes a particular gene product for which expression is desired, according to the

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normal base pairing and codon usage relationships. Thus, the coding sequence must be placed in such relationship to transcriptional control sequences (possibly including control elements and translational initiation and termination codons) that a proper length transcript will be produced and will result in translation in the appropriate reading frame to produce a functional desired product.

In a preferred embodiment the interferon alpha coding sequence is for human interferon alpha and preferably is a synthetic sequence having optimal codon usage, such as the nucleotide sequence of SEQ ID NO:11 or semi-optimal codon usage, such as the nucleotide sequence of SEQ ID NO:12.

A particular example of coding regions suitable for use in the plasmids of this invention are the natural sequences coding for human interferon alpha. Thus, in a preferred embodiment coding region has a nucleotide sequence which is the same as SEQ ID NO:10, which is the natural nucleotide sequence encoding human interferon alpha. However, it may be preferable to have an interferon alpha coding sequence which is a synthetic coding sequence. In a preferred embodiment, the interferon alpha coding sequence synthetic sequence utilizing optimal or semi-optimal codon usage, preferably the sequence shown in SEQ ID NO:11 or SEO ID NO:12.

a "sequence coding for the human interferon alpha" or "a human interferon alpha coding sequence" is a nucleic acid sequence which encodes the amino acid sequence of human interferon alpha, based on the normal base pairing translational codon usage relationships. Ιt preferable that the coding sequence encode the exact, full amino acid sequence of natural human interferon, but this is not essential. The encoded polypeptide may differ from natural human interferon alpha, so long as the polypeptide retains interferon alpha activity, preferably polypeptide is at least 50%, 75%, 90%, or 97% as active as natural human interferon alpha, and more preferably fully as

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active as natural human interferon alpha. Thus, the polypeptide encoded by the interferon alpha coding sequence may differ from a natural human interferon alpha polypeptide by a small amount, preferably less than a 15%, 10%, 5%, or 1% change. Such a change may be of one of more different types, such as deletion, addition, or substitution of one or more amino acids.

The term "transcriptional control sequence" refers to sequences which control the rate of transcription of a transcriptionally linked coding region. Thus, the term can promoters, operators, and such as include elements particular transcription unit, the enhancers. For а transcriptional control sequences will include at least a promoter sequence.

A "growth hormone 3' untranslated region" is a sequence 3') of the region encoding downstream (i.e., located material polypeptide and including at least part of the sequence of the natural 3' UTR/poly(a) signal from a growth hormone gene, preferably the human growth hormone gene. This region is generally transcribed but not translated. eukaryotic cells it is generally in expression preferable to include sequence which signals the addition of a poly-A tail. As with other synthetic genetic elements a synthetic 3' UTR/poly(A) signal has a sequence which differs from naturally-occurring UTR elements.

The sequence may be modified, for example by the deletion of ALU repeat sequences. Deletion of such ALU repeat sequences acts to reduce the possibility of homologous recombination between the expression cassette and genomic material in a transfected cell.

The plasmid preferably includes a promoter, a TATA box, a Cap site and a first intron and intron/exon boundary in appropriate relationship for expression of the coding sequence. The plasmid may also include a 5' mRNA leader sequence inserted between the promoter and the coding sequence and/or an intron/5' UTR from a chicken skeletal  $\alpha$ -

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actin gene. Also, the plasmid may have a nucleotide sequence which is the same as the nucleotide sequence of plasmid pIF0921, as shown in Figure 5.

The plasmid may also include: (a) a first transcription unit containing a first transcriptional control sequence 5 transcriptionally linked with a first 5'-untranslated region, a first intron, a first coding sequence, and a first 3'-untranslated region/poly(A) signal, wherein the intron is between the control sequence and the first coding 10 sequence; and (b) a second transcription unit containing a second transcriptional control sequence transcriptionally linked with a second 5'-untranslated region, intron, a second coding sequence, and а second untranslated region/poly(A) signal, wherein the second intron is between the control sequence and the second coding 15 sequence; wherein the first and second coding sequences contain a sequence having the sequence of SEQ ID NO:2, 3, 4 or 25 coding for a human IL-12 p40 subunit, and a sequence having the sequence of SEQ ID NO:6, 7, 8 or 24 coding for a 20 human IL-12 p35 subunit.

The term "transcription unit" or "expression cassette" refers to a nucleotide sequence which contains at least one coding sequence along with sequence elements which direct the initiation and termination of transcription. transcription unit may however include additional sequences, which may include sequences involved in post-transcriptional or post-translational processes. In preferred embodiments, the first transcriptional control sequence or the second transcriptional control sequence contain one or cytomegalovirus promoter sequences. The first and second transcriptional control sequences can be the same different.

A "5' untranslated region" or "5' UTR" refers to a sequence located 3' to promoter region and 5' of the downstream coding region. Thus, such a sequence, while transcribed, is upstream of the translation initiation codon

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and therefore is not translated into a portion of the polypeptide product.

For the plasmids described herein, one or more of a 5' untranslated promoter, region (5' UTR), UTR/poly(A) signal, and introns may be a synthetic sequence. In this context the term "synthetic" means that the sequence is not provided directly by the sequence of a naturally occurring genetic element of that type but rather is an artificially created sequence (i.e., created by a person by molecular biological methods). While one or more portions of such a synthetic sequence may be the same as portions of naturally occurring sequences, the full sequence over the specified genetic element is different from a naturally occurring genetic element of that type. The use of such genetic elements allows the functional characteristics of that element to be appropriately designed for the desired function.

Thus, a "synthetic intron" refers to a sequence which is not a naturally occurring intron sequence but which will be removed from an RNA transcript during normal post transcriptional processing. Such introns can be designed to have a variety of different characteristics, in particular such introns can be designed to have a desired strength of splice site.

A "subunit" of a therapeutic molecule is a polypeptide or RNA molecule which combines with one or more other molecules to form a complex having the relevant pharmacologic activity. Examples of such complexes include homodimers and heterodimers as well as complexes having greater numbers of subunits. A specific example of a heterodimer is IL-12, having the p40 and p35 subunits.

The "p40 subunit" is the larger of the two subunits of the IL-12 heterodimer. Thus, it is capable of association with p35 subunit to form a molecule having activities characteristic of IL-12. Human p40 has the amino acid sequence of SEQ ID NO:1. Those skilled in the art will

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recognize that the molecule may have a number of changes from that sequence, such as deletions, insertions or changes of one or a few amino acids, while still retaining IL-12 activity when associated with p35. Such active altered molecules are also regarded as p40.

Conversely, the "p35 subunit" is the smaller of the two heterodimeric subunits of IL-12. For humans, p35 has the amino acid sequence of SEQ ID NO:5. As for p40, p35 may have a low level of alterations from that sequence while still being regarded as p35.

A particular example of coding regions suitable for use in the plasmids of this invention are the natural sequences coding for the p40 and p35 subunits of human IL-12. Thus, in a preferred embodiment the first and second coding regions are coding regions for those sequences and are preferably in the order p40 then p35 in the 5' to 3' direction.

Thus, a "sequence coding for the p40 subunit of human IL-12" is a nucleic acid sequence which encodes the human p40 subunit as described above, based on the normal base pairing and translational codon usage relationships. The sequence coding for p35 subunit of human IL-12 is similarly defined.

In a preferred embodiment the sequence coding for the p40 subunit of human IL-12 is 5' to the sequence coding for the p35 subunit of human IL-12. Those skilled in the art will appreciate that the interferon alpha, p35 subunit and p40 subunit may all be on a single transcription unit, that all three may be on separate transcription units, or that any two coding sequences may be on one transcription unit and the other coding sequence on another transcription unit.

The plasmid may also contain an intron having variable splicing, a first coding sequence, and a second coding sequence, wherein the first and second coding sequences include a sequence having the sequence of SEQ ID NO:2, 3, 4 or 25 coding for a human IL-12 p40 subunit, and a sequence

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having the sequence of SEQ ID NO:6, 7, 8 or 24 coding for a human IL-12 p35 subunit.

In a preferred embodiment, the plasmid also has: (a) a transcriptional control sequence transcriptionally linked with a first coding sequence and a second coding sequence; (b) a 5'-untranslated region; (c) an intron 5' to the first coding sequence; (d) an alternative splice site 3' to the first coding sequence and 5' to the second coding sequence; and (e) a 3'-untranslated region/poly(A) signal. The transcriptional control sequence preferably includes a cytomegalovirus promoter sequence.

In a preferred embodiment, the plasmid also has: (a) a transcriptional control sequence transcriptionally linked with a first coding sequence, an IRES sequence, a second 3'-untranslated region/poly(A) sequence, and а signal, wherein the IRES sequence is between the first coding sequence and the second coding sequence; and (b) an intron between the promoter and the first coding sequence; wherein the first and second coding sequences include a sequence having the sequence of SEQ ID NO:2, 3, 4 or 25 coding for a human IL-12 p40 subunit, and a sequence having the sequence of SEQ ID NO:6, 7, 8 or 24 coding for a human The transcriptional control sequence IL-12 p35 subunit. preferably includes a cytomegalovirus promoter sequence and the IRES sequence preferably is from an encephalomyocarditis virus.

For delivery of coding sequences for gene expression, it is generally useful to provide a delivery composition or delivery system which includes one or more other components nucleic acid to the sequences. addition maintaining the in example, aid composition can, for integrity of the DNA and/or in enhancing cellular uptake of the DNA and/or by acting as an immunogenic enhancer, such as an immuno-stimulatory components having the non-DNA effect of their own.

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Thus, in another aspect, the invention features a composition containing a plasmid as described above and a protective, interactive non-condensing compound (PINC).

The PINC enhances the delivery of the nucleic acid molecule to mammalian cells in vivo, and preferably the 5 nucleic acid molecule includes a coding sequence for a gene product to be expressed in the cell. In many cases, the relevant gene product is a polypeptide or Preferably the PINC is used under conditions so that the PINC does not form a gel, or so that no gel form is present 10 at the time of administration at about  $30-40\Box C$ . Thus, in these compositions, the PINC is present at a concentration of 30% (w/v) or less. In certain preferred embodiments, the PINC concentration is still less, for example, 20% or less, 10% or less, 5% or less, or 1% or less. 15 Thus, these compositions differ in compound concentration and functional effect from uses of these or similar compounds in which the compounds are used at higher concentrations, for example in ethylene glycol mediated transfection of protoplasts, or the formation of gels for drug or nucleic 20 acid delivery. In general, the PINCs are not in gel form in the conditions in which they are used as PINCs, certain of the compounds may form gels under some conditions.

25 In connection with the compounds and compositions of this invention, the term "protects" or "protective" refers to an effect of the interaction between such a compound and a nucleic acid such that the rate of degradation of the nucleic acid is decreased in a particular environment. 30 degradation may be due to a variety of different factors, which specifically include the enzymatic action nuclease. The protective action may be provided different ways, for example, by exclusion of the nuclease molecules or by exclusion of water.

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the nucleic acid by with associate interact or intermolecular forces and/or valence bonds such as: Waals forces, ion-dipole interactions, ion-induced dipole bonds. hydrogen bonds, or ionic interactions, following functions: (1)interactions may serve the Stereoselectively protect nucleic acids from nucleases by shielding; (2) facilitate the cellular uptake of nucleic Piggyback endocytosis is acid by "piggyback endocytosis". the cellular uptake of a drug or other molecule complexed to a carrier that may be taken up by endocytosis. CV Uglea and Medical Applications of Synthetic Dumitriu-Medvichi, Polymeric Biomaterials, Severian Dumitriu Oligomers, In: Marcel Dekker, Inc., 1993, incorporated herein by ed., reference.

To achieve the desired effects set forth it is desirable, but not necessary, that the compounds which protect the nucleic acid and/or prolong the bioavailability of a nucleic acid have amphiphilic properties; that is, have both hydrophilic and hydrophobic regions. The hydrophilic region of the compounds may associate with the largely ionic and hydrophobic regions of the nucleic acid, while the hydrophobic region of the compounds may act to retard diffusion of nucleic acid and to protect nucleic acid from nucleases.

Additionally, the hydrophobic region may specifically interact with cell membranes, possibly facilitating endocytosis of the compound and thereby also of nucleic acid associated with the compound. This process may increase the pericellular concentration of nucleic acid.

Agents which may have amphiphilic properties and are generally regarded as being pharmaceutically acceptable are the following: polyvinylpyrrolidones; polyvinylalcohols; polyvinylacetates; propylene glycol; polyethylene glycols; poloxamers (Pluronics); poloxamines (Tetronics); ethylene vinyl acetates; methylcelluloses, hydroxypropylcelluloses, hydroxypropylmethylcelluloses; heteropolysaccharides

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(pectins); chitosans; phosphatidylcholines (lecithins); miglyols; polylactic acid; polyhydroxybutyric acid; xanthan Also, copolymer systems such as polyethylene glycolpolylactic acid (PEG-PLA), polyethylene polyhydroxybutyric acid (PEG-PHB), polyvinylpyrrolidonepolyvinylalcohol (PVP-PVA), and derivatized copolymers such as copolymers of N-vinyl purine (or pyrimidine) derivatives and N-vinylpyrrolidone. However, not all of the above compounds are protective, interactive, non-condensing compounds as described below.

In connection with the protective, interactive, non-condensing compounds for these compositions, the term "non-condensing" means that an associated nucleic acid is not condensed or collapsed by the interaction with the PINC at the concentrations used in the compositions. Thus, the PINCs differ in type and/or use concentration from such condensing polymers. Examples of commonly used condensing polymers include polylysine, and cascade polymers (spherical polycations).

20 Also connection in with such compounds and associated nucleic acid molecule, the term "enhances the delivery" means that at least in conditions such that the amounts of PINC and nucleic acid is optimized, a greater biological effect is obtained than with the delivery of nucleic acid in saline. Thus, in cases where the expression 25 of a gene product encoded by the nucleic acid is desired, the level of expression obtained with the PINC:nucleic acid composition is greater than the expression obtained with the same quantity of nucleic acid in saline for delivery by a method appropriate for the particular PINC/coding sequence 30 combination.

In preferred embodiments of the above compositions, the PINC is polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), a PVP-PVA co-polymer, N-methyl-2-pyrrolidone (NM2P), ethylene glycol, or propylene glycol. In compositions in which a Poloxamer (Pluronics) is used, the nucleic acid is

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preferably not a viral vector, i.e., the nucleic acid is a non-viral vector.

In other preferred embodiments, the PINC is bound with a targeting ligand. Such targeting ligands can be of a variety of different types, including but not limited to galactosyl, residues, fucosal residues, mannosyl residues, carntitine derivatives, monoclonal antibodies, polyclonal antibodies, peptide ligands, and DNA-binding proteins. The targeting ligands may bind with receptors on cells such as antigen-presenting cells, hepatocytes, myocytes, epithelial cells, endothelial cells, and cancer cells.

In connection with the association of a targeting ligand and a PINC, the term "bound with" means that the parts have an interaction with each other such that the thermodynamically favored, association is physical representing at least a local minimum in the free energy function for that association. Such interaction may involve or non-covalent interactions such as covalent binding, interactions, der Waals hydrogen bonding, van ionic, combinations of such and interactions, hvdrophobic interactions.

While the targeting ligand may be of various types, in one embodiment the ligand is an antibody. Both monoclonal antibodies and polyclonal antibodies may be utilized.

The nucleic acid may also be present in various forms. Preferably the nucleic acid is not associated with a compounds(s) which alter the physical form, however, in other embodiments the nucleic acid is condensed (such as with a condensing polymer), formulated with cationic lipids, formulated with peptides, or formulated with cationic polymers.

In preferred embodiments, the protective, interactive non-condensing compound is polyvinyl pyrrolidone, and/or the plasmid is in a solution having between 0.5% and 50% PVP, more preferably about 5% PVP. The DNA preferably is at least about 80% supercoiled, more preferably at least about

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90% supercoiled, and most preferably at least about 95% supercoiled.

In another aspect the invention features a composition containing a protective, interactive non-condensing compound and a plasmid containing an interferon alpha coding sequence.

In yet another aspect, the invention provides a composition containing a plasmid of the invention (or a plasmid containing an interferon alpha coding sequence) and a cationic lipid with a neutral co-lipid.

Preferably the cationic lipid is DOTMA and the neutral co-lipid is cholesterol (chol). DOTMA is 1,2-di-0octadecenyl-3-trimethylammonium propane, which is described and discussed in Eppstein et al., U.S. Patent 4,897,355, issued January 30, 1990, which is incorporated herein by 15 However, other lipids and lipid combinations may reference. be used in other embodiments. A variety of such lipids are described in Gao & Huang, 1995, Gene Therapy 2:710-722, which is hereby incorporated by reference.

As the charge ratio of the cationic lipid and the DNA is also a significant factor, in preferred embodiments the DNA and the cationic lipid are present is such amounts that the negative to positive charge ratio is about 1:3. While preferable, it is not necessary that the ratio be 1:3.

Thus, preferably the charge ratio for the compositions is between about 1:1 and 1:10, more preferably between about 1:2 and 1:5.

The term "cationic lipid" refers to a lipid which has a net positive charge at physiological pH, and preferably carries no negative charges at such pH. An example of such a lipid is DOTMA. Similarly, "neutral co-lipid" refers to a lipid which has is usually uncharged at physiological pH. An example of such a lipid is cholesterol.

Thus, "negative to positive charge ratio" for the DNA and cationic lipid refers to the ratio between the net

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negative charges on the DNA compared to the net positive charges on the cationic lipid.

the DNA affects the form of the efficiency, the DNA preferably is at least about supercoiled, more preferably at least about 90% supercoiled, and most preferably at least about 95% supercoiled. composition preferably includes an isotonic carbohydrate solution, such as an isotonic carbohydrate solution that consists essentially of about 10% lactose. In preferred embodiments, the composition the cationic lipid and the neutral co-lipid are prepared as a liposome having an extrusion size of about 800 nanometers. Preferably the liposomes are prepared to have an average diameter of between about 20 and 800 nm, more preferably between about 50 and 400 nm, still more preferably between about 75 and 200 nm, and most preferably about 100 nm. Microfluidization is the preferred method of preparation of the liposomes.

In another aspect the invention features a composition containing: (a) a first component having a plasmid including an interferon alpha coding sequence and a cationic lipid with a neutral co-lipid, wherein the cationic lipid is DOTMA and the neutral co-lipid is cholesterol, wherein the DNA in the plasmid and the cationic lipid are present in amounts such that the negative to positive charge ratio is about 1:3; and (b) a second component including a protective, interactive non-condensing compound, wherein the first component is present within the second component.

In another aspect, the invention provides a composition having a protective, interactive non-condensing compound, a first plasmid including an interferon alpha coding sequence, and one or more other plasmids independently having an IL-12 p35 or IL-12 p40 subunit coding sequence.

In another aspect, the invention features a method for making any of the plasmids described above by inserting a CMV promoter transcriptionally linked with an interferon

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alpha coding sequence, and a growth hormone 3'-untranslated region into a plasmid.

The invention also provides methods of making the compositions described above. The method may involve: (a) preparing a DNA molecule having a transcriptional unit, wherein the transcriptional unit contains an interferon alpha coding sequence; (b) preparing a protective, interactive non-condensing compound; and (c) combining the protective, interactive non-condensing compound with the DNA in conditions such that a composition capable of delivering a therapeutically effective amount of an interferon alpha coding sequence to a mammal is formed.

Preferably, the DNA molecule is a plasmid, wherein the plasmid includes a CMV promoter transcriptionally linked with an interferon alpha coding sequence, and a human growth hormone 3'-untranslated region/poly(A) signal.

The method may involve the steps of: (a) preparing a DNA having an interferon alpha coding sequence; (b) preparing a mixture of a cationic lipid and a neutral colipid, wherein the cationic lipid is DOTMA and the neutral co-lipid is cholesterol; and (c) combining the mixture with the DNA in amounts such that the cationic lipid and the DNA are present in a negative to positive charge ratio of about 1:3.

25 In another embodiment, the method involves the steps preparing a first component having a plasmid (a) containing an interferon alpha coding sequence cationic lipid with a neutral co-lipid, wherein the cationic lipid is DOTMA and the neutral co-lipid is cholesterol, wherein the DNA in the plasmid and the cationic lipid are 30 present in amounts such that the negative to positive charge ratio is about 1:3; (b) preparing a second component having a protective, interactive non-condensing compound; and (c) combining the first and second components such that the resulting composition includes the first component within 35 the second component.

In another embodiment, the method involves the steps of: (a) preparing a protective, interactive non-condensing compound, (b) preparing a first plasmid having an interferon alpha coding sequence, (c) preparing one or more other plasmids independently having an IL-12 p35 or IL-12 p40 subunit coding sequence, and (d) combining the protective, interactive non-condensing compound, the plasmid having the interferon alpha coding sequence and the other plasmids.

In another aspect, the invention provides a method for treatment of a mammalian condition or disease, by administering to a mammal suffering from the condition or disease a therapeutically effective amount of a plasmid as described herein.

A "therapeutically effective amount" of a composition is an amount which is sufficient to cause at least temporary relief or improvement in a symptom or indication of a disease or condition. Thus, the amount is also sufficient The amount of the to cause a pharmacological effect. permanent improvement not cause need composition indications. symptoms or all of improvement therapeutically effective amount of a cancer therapeutic would be one that reduces overall tumor burden in the case of metastatic disease (i.e., the number of metasteses or their size) or one that reduces the mass of the tumor in localized cancers.

The condition or disease preferably is a cancer, such epithelial glandular cancer, including adenoma adenocarcinoma; squamous and transitional cancer, including squamous cell and transitional polyp, papilloma, carcinoma; connective tissue cancer, including tissue type sarcoma and other (oma's); hematopoietic positive, including lymphoma, leukemia lymphoreticular cancer, Hodgkin's disease; neural tissue cancer, including neuroma, sarcoma, neurofibroma and blastoma; mixed tissues of origin cancer, including teratoma and teratocarcinoma. Other applicable to treatment cancerous conditions that are

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include cancer of any of the following: adrenal gland, anus, bile duct, bladder, brain tumors: adult, breast, cancer of an unknown primary site, carcinoids of the gastrointestinal tract, cervix, childhood cancers, colon and esophagus, gall bladder, head and neck, islet cell and other pancreatic carcinomas, Kaposi's sarcoma, kidney, leukemia, liver, lung: non-small cell, lung: small cell, lymphoma: AIDS-associated, lymphoma: Hodgkin's disease, Lymphomas: non-Hodgkin's disease, melanoma, mesothelioma, metastatic cancer, multiple myeloma, ovary, ovarian germ cell tumors, 10 pancreas, parathyroid, penis, pituitary, prostate, sarcomas of bone and soft tissue, skin, small intestine, stomach, testis, thymus, thyroid, trophoblastic disease, endometrial carcinoma, uterus: uterine sarcomas, vagina, or 15 The composition preferably is administered injection, although the method may also be performed vivo.

In another aspect, the invention provides a method for transfection (i.e., the delivery and expression of a gene to cells) of a cell in situ, by contacting the cell with a plasmid described herein for sufficient time to transfect the cell. Transfection of the cell preferably is performed in vivo and the contacting preferably is performed in the presence of about 5% PVP solution.

In another aspect, the invention features a method for delivery and expression of an interferon alpha gene in a plurality of cells, by: (a) transfecting the plurality of cells with a plasmid or composition of the invention; and (b) incubating the plurality of cells under conditions allowing expression of a nucleic acid sequence in the vector, wherein the nucleic acid sequence encodes interferon alpha.

In preferred embodiments, the interferon alpha is human interferon alpha and the cells are human cells and/or the contacting is performed in the presence of an about 5% PVP solution.

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In another aspect, the invention features a method for treating a disease or condition, by transfecting a cell in situ with a plasmid or composition of the invention. The disease or condition can be a localized disease or condition or a systemic disease or condition.

In another aspect, the invention features a cell transfected with a plasmid or composition of the invention.

In yet another aspect, the invention features a method for treatment of a mammalian condition or disease, by administering to a mammal suffering from the condition or disease a therapeutically effective amount of a composition described herein.

As the compositions are useful for delivery of a nucleic acid molecule to cells in vivo, in a related aspect the invention provides a composition at an in vivo site of administration. In particular this includes at an in vivo site in a mammal.

In preferred embodiments the nucleic acid molecule includes a sequence encoding a gene product. Also in preferred embodiments, the site of administration is in an interstitial space or a tissue of an animal, particularly of a mammal.

The invention also provides methods for using the above compositions. Therefore, in further related aspects, methods of administering the compositions are provided in which the composition is introduced into a mammal, preferably into a tissue or an interstitial space.

Various methods of delivery may be utilized, such as are known in the art, but in preferred embodiments, the composition is introduced into the tissue or interstitial space by injection. The compositions may also be delivered to a variety of different tissues, but in preferred embodiments the tissue is muscle or tumor.

In another related aspect, the invention provides 35 methods for treating a mammalian condition or disease by administering a therapeutically effective amount of a

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composition as described above. In preferred embodiments, the disease or condition is a cancer.

The summary of the invention described above is nonlimiting and other features and advantages of the invention will be apparent from the following detailed description of the preferred embodiments, as well as from the claims.

# Brief Description Of The Drawings

Figure 1 shows the effects of interferon alpha in two cancer models.

10 Figure 2 shows a plasmid map and sequence (SEQ ID NO:18) for an exemplary IL-12 plasmid of the present invention.

Figure 3 shows optimal codon usage for highly expressed human genes.  $\ \ \,$ 

Figure 4 shows a plasmid map and sequence (SEQ ID NO:19) for plasmid pIF0836, an exemplary interferon alpha plasmid of the present invention.

Figure 5 shows a plasmid map and sequence (SEQ ID NO:20) for pIN096, an exemplary IL-12 plasmid that can be used with the present invention.

Figure 6 shows the nucleic acid sequence (SEQ ID NO:21) of plasmid pIF0921, an exemplary interferon alpha plasmid of the present invention.

Figures 7A and 7B show a plasmid map and sequence (SEQ 25 ID NO:22) for plasmid pIF0921.

Figure 8 shows an outline of a strategy that can be used to synthesize a pIF0921 plasmid.

Figure 9 shows interferon alpha and IL-12 gene medicine (combination therapy) in Renca model.

# 30 Detailed Description Of The Preferred Embodiments

The present invention relates to gene delivery and gene therapy, and provides novel nucleic acid constructs for expression of interferon alpha in a mammal, formulations for delivery that incorporate a nucleic acid construct for

expression, and methods for preparing and using such constructs and formulations. In particular, this invention relates to plasmid constructs for delivery of therapeutic interferon alpha encoding nucleic acids to cells in order to modulate tumor activity, methods of using those constructs (including combination therapy with other agents, such as cytokines, preferably IL-12), as well as methods for preparing such constructs.

#### I. General

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As described, this invention concerns expression systems for the delivery and expression of interferon alpha coding sequences in mammalian cells, and formulations and methods for delivering such expression systems or other expression systems to a mammal.

Therefore, particular genetic constructs are described which includes nucleotide sequences coding for interferon alpha, preferably human interferon alpha. Such a construct can beneficially be formulated and administered as described herein, utilizing the expression systems of this invention.

To allow convenient production of such plasmids, it is generally preferable that the plasmid be capable of replication in a cell to high copy number. Generally such production is carried out in prokaryotic cells, particularly including Esherichia coli (E.coli) cells. Thus, the plasmid preferably contains a replication origin functional in a prokaryotic cell, and preferably the replication origin is one which will direct replication to a high copy number.

It is also possible to utilize synthetic genetic elements in the plasmid constructs.

As described below, these elements affect posttranscriptional processing in eukaryotic systems. Thus, the use of synthetic sequences allows the design of processing characteristics as desired for the particular application. Commonly, the elements will be designed to provide rapid and accurate processing.

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For delivery of DNA encoding a desired expression product to a mammalian system, it is usually preferable to utilize a delivery system. Such a system can provide multiple benefits, notably providing stabilization to protect the integrity of the DNA, as well as assisting in cellular uptake.

In addition, the non-DNA components of the formulation may contribute to an immune system enhancement activation. As a result, components of a delivery system selected in conjunction with a particular gene product to enhance or minimize the immuno-stimulatory effect.

The plasmids may also include elements for expression of IL-12 or one or more subunits thereof. Similarly, the treatment may involve administration of an interferon alpha coding sequence and one or more IL-12 coding sequences whether on a single plasmid or on separate plasmids. Such plasmids may be incorporated into compositions for delivery with a protective, interactive non-condensing compound, a cationic lipid and neutral co-lipid, or both.

While these are specific effective examples, other components may be utilized in formulations containing the interferon alpha expression vectors of the present invention to provide effective delivery and expression of interferon alpha or with other coding sequences for which manipulation of the activation of immune system components is desirable.

The selection of delivery system components and preparation methods in conjunction with the selection of coding sequences provides the ability to balance the specific effects of the encoded gene products and the immune system effects of the overall delivery system composition. This capacity to control the biological effects of delivery system formulation administration represents an aspect of the invention in addition to the interferon alpha encoding constructs and specific formulations of delivery systems. Co-selection of the encoded gene product and the delivery

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system components and parameters provides enhanced desired effects rather than merely providing high level gene expression. In particular, such enhancement is described below for the antitumor effects of the exemplary PVP containing compositions.

For systems in which IL-12 is also administered, the antitumor effect can be greater than merely additive (i.e., greater than merely the sum of the antitumor effects of interferon alpha alone and IL-12 alone). Enhancement of immuno-stimulatory effects is also desirable in other contexts, for example, for vaccine applications.

In contrast, for certain applications, it is preferable to select a delivery systems which minimizes the immune system effects. For example, it is often preferred that the immune system activation be minimized for compositions to be delivered to the lung in order to minimize lung tissue swelling.

A useful approach for selecting the delivery system components and preparation techniques for a particular coding sequence can proceed as follows, but is not limited to these steps or step order.

- Select a particular genetic construct which provides appropriate expression in vitro.
- 2. Select delivery system components based on desired immunostimulatory effects and/or on in vivo physiological effect. Such effects can be tested or verified in in vivo model systems.
- 3. Select the other delivery system parameters consistent with the desired immuno-stimulatory effects and/or consistent with enhancing the desired in vivo physiological effect. Such parameters can, for example, include the amount and ratio of DNA to one or more other composition components, the relative amounts of non-DNA composition components, the size of delivery system formulation particles, the percent

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supercoiled DNA for circular dsDNA vectors, and the specific method of preparation of delivery system formulation particles. The particular parameters relevant for specific types of formulations will be apparent or readily determined by testing.

The description below illustrates the selection of components and parameters in the context of interferon alpha encoding constructs. However, it should be recognized that the approach is applicable to constructs containing a variety of other coding sequences.

# II. Plasmid Construct Expression Systems

## A. Plasmid Design and Construction

For the methods and constructs of this invention, a number of different plasmids were constructed which are useful for delivery and expression of sequences encoding interferon alpha. Thus, these plasmids contain coding regions for interferon alpha, along with genetic elements necessary or useful for expression of those coding regions.

While these embodiments utilized into 5.

While these embodiments utilized interferon alpha cDNA clones or partial genomic sequences from a particular source, those skilled in the art could readily obtain interferon alpha coding sequences from other sources, or obtain a coding sequence by identifying a cDNA clone in a library using a probe(s) based on the published interferon alpha coding and/or flanking sequences. This also applies to the IL-12 coding sequences utilized in the embodiments described herein.

Coding sequences for interferon alpha were incorporated into an expression plasmid that contains eukaryotic and bacterial genetic elements. Eukaryotic genetic elements include the CMV immediate early promoter and 5' UTR, and a human growth hormone 3' UTR/poly(a) signal, which influence gene expression by controlling the accuracy and efficiency of RNA processing, mRNA stability, and translation.

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The human growth hormone 3' UTR is from a human growth hormone gene, and preferably includes a poly(a) signal. This sequence can be linked immediately following the natural translation termination codon for a cDNA sequence, genomic sequence, modified genomic sequence, or synthetic sequence coding for interferon alpha.

An example of a human growth hormone 3' UTR/poly(a) signal is shown by the human growth hormone 3' UTR (nucleotides 1 - 100) and 3' flanking sequence (nucleotides 101 - 191; GenBank accession #J03071, HUMGHCSA) below. (SEQ ID NO:13)

- 1 GGGTGGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGT Poly (a)signal
- 51 TGCCACTCCAGTGCCCACCAGCCTTGTCCTAATAAAATTAAGTTGCATCA
- 101 TTTTGTCTGACTAGGTGTCCTTCTATAATATTATGGGGTGGAGGGGGGTG
- 20 151 GTATGGAGCAAGGGGCAAGTTGGGAAGACAACCTGTAGGGC

The 5' and 3' UTR and flanking regions can be further and more precisely defined by routine methodology, e.g., deletion or mutation analysis or their equivalents., and can be modified to provide other sequences having appropriate transcriptional and translational functions.

1. Construction of plasmid: Plasmid Backbone, human interferon alpha cDNA, Final Construct

A diagrammatic representation of the PCR products and plasmids involved in creation of an exemplary construct is shown below in Figure 8.

Plasmid pIF0921 was constructed from commercially available plasmids, and contains the TN5 gene encoding the kanamycin resistance gene, the pUC origin of replication, the CMV enhancer and promoter to base +112, a synthetic intron called IVS8, the human IFN-a2b gene, and the human growth hormone 3' UTR. The plasmid construction descendancy

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for pIL0697 is shown in Figure 8. pIL0697 was cut with BamHI and Xba I and the hIFN-a2b PCR product, which had been amplified from human genomic DNA with BamHI and Xba I ends, was cloned into the pIL0697 backbone in place of the IL-2 coding region. The resulting plasmid was pIF0863. pIF0863 was cut with Nco I and intron IVS8 from pCT0828 was cloned in. The resulting plasmid was pIF0890. pIF0890 was cut with Nde I and Pac I and an additional region of the CMV 5' UTR to base +112 was cloned in from plasmid pLC0888.

# 10 B. Synthetic Genetic Elements

some embodiments, some or all of the genetic elements can be synthetic, derived from synthetic oligonucleotides, and thus are not obtained directly from natural genetic sequences. These synthetic elements are appropriate for use in many different expression vectors.

A synthetic intron is designed with splice sites that ensure that RNA splicing is accurate and efficient. A synthetic 3' UTR/poly(A) signal is designed to facilitate mRNA 3' end formation and mRNA stability. A synthetic 5' UTR is designed to facilitate the initiation of translation. The design of exemplary synthetic elements is described in more detail below.

# 1. Summary of Synthetic Element Features Exemplary synthetic 5'UTR, intron, and 3'UTR/poly(A)

25 signal have the general features shown below:

5' UTR Short.

Lack of secondary structure. Kozak sequence.

Site for intron insertion.

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Intron

5' splice site sequence matches consensus.
5' splice site sequence is exactly complementary to 5' end of U1 snRNA.
Branch point sequence matches consensus.
Branch point sequence is complementary to U2 snRNA.
3' splice site matches consensus.
Polypyrimidine tract is 16 bases i

Polypyrimidine tract is 16 bases in length and contains 7 consecutive T's. (The tract preferably contains at least 5 consecutive T's.)

Contains internal restriction enzyme sites.

BbsI cleaves at the 5'ss, EarI

cleaves at the 3'ss.

3' UTR/Poly(A) Based on rabbit  $\beta$ -globin 3' UTR/poly(A) signal. Consists of two poly(A) signals in tandem.

#### 2. Features of the Synthetic 5'UTR (UT6):

The 5' untranslated region (5'UTR) influences the translational efficiency of messenger RNA, and is therefore an important determinant of eukaryotic gene expression. The synthetic 5'UTR sequence (UT6) has been designed to maximize the translational efficiency of mRNAs encoded by vectors that express genes of therapeutic interest.

The sequence of the synthetic 5' UTR (UT6) is shown below. The Kozak sequence is in boldface and the initiation codon is double underlined. The location of the intron (between residues 48 and 49) is indicated by the filled

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triangle and the sequences that form the exonic portion of consensus splice sites are single underlined. The restriction sites for HindIII and NcoI are overlined. (SEQ ID NO:14)

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The 5' untranslated region (5' UTR), located between the cap site and initiation codon, is known to influence the 10 efficiency of mRNA translation. Any features that influence the accessibility of the 5' cap structure to initiation factors, the binding and subsequent migration of the 43S preinitiation complex, or the recognition of the initiation codon, will influence mRNA translatability. An efficient 5' 15 UTR is expected to be one that is moderate in length, devoid secondary structure, devoid of upstream codons, and has an AUG within an optimal local context (Kozak, 1994, Biochimie 76:815-821; Jansen et al., 1994). A 5' UTR with these characteristics should allow efficient 20 recognition of the 5' cap structure, followed by rapid and unimpeded ribosome scanning by the ribosome, facilitating the translation initiation process.

The sequence of the synthetic 5'UTR was designed to be moderate in length (54 nucleotides (nts)), to be deficient in G but rich in C and A residues, to lack an upstream ATG, to place the intended ATG within the context of a optimal Kozak sequence (CCACCATGG), and to lack potential secondary structure. The synthetic 5' UTR sequence was also designed to lack AU-rich sequences that target mRNAs to be rapidly degraded in the cytoplasm.

Experiments have demonstrated that introns increase gene expression from cDNA vectors, and that introns located in the 5' UTR are more effective than ones located in the 3' UTR (Huang and Gorman, 1990, Mol. Cell. Biol. 10:1805-1810; Evans and Scarpulla, 1989, Gene 84:135-142; Brinster et al.,

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1988, Proc. Natl. Acad. Sci. USA 85:836-840; Palmiter et al., 1991, Proc. Natl. Acad. Sci. USA 88:478-482; Choi et al., 1991, Mol. Cell. Biol. 11:3070-3074). Accordingly, the synthetic 5' UTR sequence was designed to accommodate an intron with consensus splice site sequences. The intron may, for example, be located between residues 48 and 49 (See intron sequence structure below). The CAG at position 46-48 is the exonic portion of a consensus 5' splice site. The G at position 49 is the exonic portion of a consensus 3' splice site.

To facilitate cloning manipulations, the synthetic 5' UTR sequence was designed to begin with a HindIII site and terminate with a NcoI site.

#### 3. Features of the Synthetic Intron

RNA splicing is required for the expression of most eukaryotic genes. For optimal gene expression, RNA splicing must be highly efficient and accurate. A synthetic intron, termed OPTIVS8B, was designed to be maximally efficient and accurate.

the exemplary synthetic intron, The structure of 20 Sequences for the 5' splice site OPTIVS8 is shown below. (5'ss), branch point (bp), and 3' splice site (3'ss) The recognition sequences for the double underlined. restriction enzymes BbsI and EarI are overlined. The cleavage site for BbsI corresponds to the 5'ss, and the 25 cleavage site for EarI corresponds to the 3'ss.

5'ss bp 3'ss

| BbsI | EarI |

30 5'CAG GTAAGTGTCTTC---(77)---TACTAACGGTTCTTTTTTCTCTCACAG G 3'

(SEQ ID NO.15) (SEQ ID NO.16)

The 5' splice site (5'ss) sequence matches the established consensus sequence, MAG  $\square$  GTRAGT, where M = C or A, and R = G or A. Since the mechanism of splicing involves an interaction between the 5'ss of the pre-mRNA and U1

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snRNA, the 5'ss sequence of OPTIVS8B was chosen to be exactly complementary to the 5' end of Ul snRNA.

5'ss 5' CAGGUAAGU 3'
||||||||
U1 RNA 3' GUCCAUUCA 5'

In mammals, the consensus sequence for branch points (YNYTRAY, where Y = C or T, R = A or G, N = any base, and the underlined A residue is the actual branch point) is very ambiguous. Since the mechanism of splicing involves an interaction between the branch point (bp) of the pre-mRNA and U2 snRNA, the branch point sequence of OPTIVS8B was chosen to maximize this interaction. (Note that the branch point itself is bulged out). The chosen sequence also matches the branch point sequence that is known to be obligatory for pre-mRNA splicing in yeast. The branch point is typically located 18-38 nts upstream of the 3' splice site. In OPTIVS8B, the branch point is located 24 nts upstream from the 3' splice site.

5' UACUAAC 3'
| | | | | | |
U2 RNA 3' AUGAU G 5'

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The sequence of the 3' splice site (3'ss) matches the established consensus sequence,  $Y_{11}NYAG \downarrow G$ , where Y = C or T, and N = any base. In 3' splice sites, the polypyrimidine tract  $(Y_{11})$  is the major determinant of splice site strength. For optimal splice site function in OPTIVS8B, the length of the polypyrimidine tract was extended to 16 bases, and its sequence was adjusted to contain 7 consecutive T residues. This feature was included because Roscigno et al. (1993) demonstrated that optimal splicing requires the presence of at least 5 consecutive T residues in the polypyrimidine tract.

Splicing in vitro is generally optimal when introns are >80 nts in length (Wieringa, et al., 1984; Ulfendahl et al., 1985, Nucl. Acids Res. 13:6299-6315). Although many introns may be thousands of bases in length, most naturally occurring introns are 90-200 nt in length (Hawkins, 1988, Nucl. Acids Res. 16:9893-9908). The length of the synthetic intron (118 nts) falls within this latter range.

OPTIVS8B was designed with three internal restriction enzyme sites, BbsI, NheI, and Earl. These restriction sites facilitate the screening and identification of genes that contain the synthetic intron sequence. In addition, BbsI and EarI sites were placed so that their cleavage sites exactly correspond to the 5'ss (BbsI) or 3'ss (EarI). polypyrimidine tract was specifically the sequence of EarI restriction the accommodate designed to Inclusion of the BbsI and EarI sites at these locations is useful because they permit the intron to be precisely deleted from a gene. They also permit the generation of an "intron cassette" that can be inserted at other locations within a gene.

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The 77 bases between the BbsI site and the branch point sequence are random in sequence, except for the inclusion of the NheI restriction site.

# 4. <u>Features of the Synthetic 3' UTR/poly(A)</u> Signal:

The 3' ends of eukaryotic mRNAs are formed by the process of polyadenylation. This process involves site specific site RNA cleavage, followed by addition of a poly(A) tail. RNAs that lack a poly(A) tail are highly unstable. Thus, the efficiency of cleavage/polyadenylation is a major determinant of mRNA levels, and thereby, of gene expression levels. 2XPA1 is a synthetic sequence, containing two efficient poly(A) signals, that is designed to be maximally effective in polyadenylation.

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A poly(A) signal is required for the formation of the 3' end of most eukaryotic mRNA. The signal directs two RNA processing reactions: site-specific endonucleolytic cleavage of the RNA transcript, and stepwise addition of adenylates (approximately 250) to the newly generated 3' end to form the poly(A) tail. A poly(A) signal has three parts: hexanucleotide, cleavage site, and downstream element. hexanucleotide is typically AAUAAA and cleavage sites are most frequently 3' to the dinucleotide CA (Sheets et al., Downstream elements are required for optimal poly(A) signal function and are located downstream of the cleavage The sequence requirement for downstream elements is not yet fully established, but is generally viewed as UG- or U-rich sequences (Wickens, 1990; Proudfoot, 1991, 64:671-674; Wahle, 1992, Bioessays 14:113-118; Chen Nordstrom, 1992, Nucl. Acids Res. 20:2565-2572).

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Naturally occurring poly(A) signals are highly variable in their effectiveness (Peterson, 1992). The effectiveness of a particular poly(A) signal is mostly determined by the quality of the downstream element. (Wahle, 1992). In expression vectors designed to express genes of therapeutic interest, it is important to have a poly(A) signal that is as efficient as possible.

Poly(A) efficiency is important for gene expression, 25 because transcripts that fail to be cleaved polyadenylated are rapidly degraded in the compartment. In fact, the efficiency of polyadenylation in living cells is difficult to measure, nonpolyadenylated RNAs are so unstable. In addition to being required for mRNA stability, poly(A) tails contribute 30 to the translatability of mRNA, and may influence other RNA processing reactions such as splicing or RNA transport ((Jackson and Standart, 1990, Cell 62:15-24; Wahle, 1992).

Some eukaryotic genes have more than one poly(A) site, 35 implying that if the cleavage/polyadenylation reaction fails to occur at the first site, it will occur at one of the

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later sites. In COS cell transfection experiments, a gene with two strong poly(A) sites yielded approximately two-fold more mRNA than one with a single strong poly(A) site (Bordonaro, 1995). These data suggest that a significant fraction of transcripts remain unprocessed even with a single "efficient" poly(A) signal. Thus, it may be preferable to include more than one poly(A) site.

The sequence of the exemplary synthetic poly(A) signal named 2XPA. The sequence is below. shown hexanucleotide sequences and downstream element sequences are double underlined, and the two poly(A) sites are labeled Convenient restriction sites pA#1 and pA#2. The entire 2XPA unit may be transferred in overlined. cloning experiments as a XbaI-KpnI fragment. Deletion of the internal BspHI fragment results in the formation of a 1XPA unit. (SEQ ID NO. 17)

XbaI BspHI

TCTAGAGCATTTTTCCCTCTGCCAAAAATTATGGGGACATCATGAAGCCCCTTGAGCATCTGACG

20 pA#1

BspHI

25 CGGTACTAGAGCATTTTTCCCTCTGCCAAAAATTATGGGGACATCATGAAGCCCCTTGAGCATCT
pA#2

30 <u>KpnI</u> CACTCGGTACC

The sequence of the synthetic poly(A) site shown above is based on the sequence of the rabbit □-globin poly(A) signal, a signal that has been characterized in the literature as strong (Gil and Proudfoot, 1987, Cell 49:399-

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406; Gil and Proudfoot, 1984, *Nature* 312:473-474). One of its key features is the structure of its downstream element, which contains both UG- and U-rich domains.

A double-stranded DNA sequence corresponding to the 1XPA sequence was constructed from synthetic oligonucleotides. Two copies of the 1XPA sequence were then joined to form the 2XPA sequence. The sequences were joined in such as way as to have a unique XbaI site at the 5' end of the first poly(A) signal containing fragment, and a unique KpnI site at the 3' end of the second poly(A) signal containing fragment.

### C. Interferon Alpha and IL-12 Coding Sequences

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The nucleotide sequence of a natural human interferon alpha coding sequences is known, and is provided below, along with a synthetic sequence which also codes for human interferon alpha. The same applies with respect to the IL-12 coding sequences.

In some cases, instead of the natural sequence coding interferon alpha, it is advantageous to synthetic sequences which encode interferon alpha. Such synthetic sequences have alternate codon usage from the natural sequence, and thus have dramatically different sequences from the natural nucleotide sequence. particular, synthetic sequences can be used which have codon usage at least partially optimized for expression in a The natural sequences do not have such optimal codon usage. Preferably, substantially all the codons optimized.

Optimal codon usage in humans is indicated by codon usage frequencies for highly expressed human genes, as shown 30 The codon usage chart is from the program in Fig. 3. "Human High.cod" from the Wisconsin Sequence Package, Version 8.1, Genetics Computer Group, Madison, WI. codons which are most frequently used in expressed human genes are presumptively the optimal codons 35

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for expression in human host cells, and thus form the basis for constructing a synthetic coding sequence. An example of a synthetic interferon alpha coding sequence is shown as the bottom sequence in the table below.

However, rather than a sequence having fully optimized codon usage, it may be desirable to utilize an interferon alpha encoding sequence which has optimized codon usage except in areas where the same amino acid is too close together or abundant to make uniform codon usage optimal.

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In addition, other synthetic sequences can be used which have substantial portions of the codon usage optimized, for example, with at least 50%, 70%, 80% or 90% optimized codons as compared to a natural coding sequence. Other particular synthetic sequences for interferon alpha can be selected by reference to the codon usage chart in Fig. 3. A sequence is selected by choosing a codon for each of the amino acids of the polypeptide sequences. molecules corresponding to each of the polypeptides can then by constructed by routine chemical synthesis methods. For example, shorter oligonucleotides can be synthesized, and then ligated in the appropriate relationships to construct the full-length coding sequences.

The following sequences are provided in the sequence listing herein: interferon alpha amino acid sequence, SEQ interferon alpha wild type nucleic acid sequence, ID NO:9; ID NO:10; interferon alpha synthetic nucleic acid codon usage, SEO ID sequence with optimized interferon alpha nucleic acid sequence with additional/semioptimized codon usage, SEQ ID NO:12; IL-12 p40 subunit amino acid sequence, SEQ ID NO:1; IL-12 p40 wild type nucleic acid sequence, SEQ ID NO:2; IL-12 p40 synthetic nucleic acid sequence with all codons optimized, SEQ ID NO:3; IL-12 p40 subunit nucleic acid sequence with all codons optimized except when same nucleic acids were too close/abundant, SEQ ID NO:4; IL-12 p35 amino acid sequence, SEQ ID NO:5; IL-12 p35 wild type nucleic acid sequence, SEQ ID NO:6; IL-12 p35

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synthetic nucleic acid sequence with all codons optimized, SEQ ID NO:7; IL-12 p35 subunit nucleic acid sequence with all codons optimized except when same nucleic acids were too close/abundant, SEQ ID NO:8. Those skilled in the art will realize that various nucleic acid sequences with optimized codon usage can be constructed, for example based on the various combinations shown below, wherein optimal usage for each codon is shown below the IL-12 p35 and p40 subunit wild type sequences and the interferon alpha wild type sequence.

### 10 Sequences Encoding Human IL-12 p35

First line = natural sequence (SEQ ID NO. 6)
Second line = all codons optimized (SEQ ID NO. 7)

Third line = all codons optimized except when same nucleic acids were too close/abundant (changes between second and third lines bolded) (SEQ ID NO. 8)

ATG TGT CCA GCG CGC AGC CTC CTC CTT GTG GCT ACC CTG GTC CTC CTG GAC CAC CTC ACT
ATG TGC CCC GCC CGC AGC CTG CTG CTG CTG GTG GCC ACC CTG GTG CTG CTG GAC CAC CTC AGC

TTG GCC AGA AAC CTC CCC GTG GCC ACT CCA GAC CCA GGA ATG TTC CCA TGC CTT CAC CAC CTG GCC GCC AAC CTG CCC GTG GCC ACC CCA GAC CCC GGC ATG TTC CCA TGC CTG CAC CAC CTG GCC GCC AAC CTC CCC GTG GCC ACC CCA GAC CCC GGC ATG TTC CCA TGC CTG CAC CAC

25 TCC CAA AAC CTG CTG AGG GCC GTC AGC AAC ATG CTC CAG AAG GCC AGA CAA ACT CTA GAA AGC CAG AAC CTG GCG GCC GTG AGC AAC ATG CTG CAG AAG GCC GCG CAG ACC CTG GAG AGC CAG AAC CTG GCG CAG AAC CTG GAG AGC CAG AAC CTG GCG CAG AAC CTG GAG

TTT TAC CCT TGC ACT TCT GAA GAG ATT GAT CAT GAA GAT ATC ACA AAA GAT AAA ACC AGC 30 TTC TAC CCC TGC ACC AGC GAG GAG ATC GAC CAC GAG GAC ATC ACC AAG GAC AAG ACC AGC TCC TAC CCC TGC ACC AGC GAG ATC GAC CAC GAG GAC ATC ACC AAG GAC AAG ACC AGC

ACA GTG GAG GCC TGT TTA CCA TTG GAA TTA ACC AAG AAT GAG AGT TGC CTA AAT TCC AGA
ACC GTG GAG GCC TGC CTG CCC CTG GAG CTG ACC AAG AAC GAG AGC TGC CTG AAC AGC CGC

35 ACC GTG GAG GCC TGC CTG CCC CTC GAG TTA ACC AAG AAC GAG AGC TGC CTC AAC AGC CGC

GAG	ACC	TCT	TTC	ATA	ACT	AAT	GGG	AGT	TGC	CTG	GCC	TCC	AGA	AAG	ACC	TCT	TTT	ATG	ATG
GAG	ACC	AGC	TTC	ATC	ACC	AAC	GGC	AGC	TGC	CTG	GCC	AGC	CGC	AAG	ACC	AGC	TTC	ATG	ATG
GAG	ACC	TCC	TTC	ATC	ACC	AAC	GGC	ACT	TGC	CTG	GCC	TCC	CGC	AAG	ACC	AGC	TTC	ATG	ATG

GCC CTG TGC CTT AGT AGT ATT TAT GAA GAC TTG AAG ATG TAC CAG GTG GAG TTC AAG ACC GCC CTG TGC CTG AGC AGC ATC TAC GAG GAC CTG AAG ATG TAC CAG GTG GAG TTC AAG ACC GCC CTG TGC CTG AGC TCC ATC TAC GAG GAC CTG AAG ATG TAC CAG GTG GAG TTC AAG ACC

10

ATG AAT GCA AAG CTT CTG ATG GAT CCT AAG AGG CAG ATC TTT CTA GAT CAA AAC ATG CTG ATG AAC GCC AAG CTG CTG ATG GAC CCC AAG CTC CAG ATC TTC CTG-GAC CAG AAC ATG CTG ATG AAC GCC AAG CTC CTG ATG GAC CCC AAG CTC CAG ATC TTC CTG GAC CAG AAC ATG CTG

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GCA GTT ATT GAT GAG CTG ATG CAG GCC CTG AAT TTC AAC AGT GAG ACT GTG CCA CAA AAA GCC GTG ATC GAC GAG CTG ATG CAG GCC CTG AAC TTC AAC AGC GAG ACC GTG CCC CAG AAG GCC GTG ATC GAC GAG CTG ATG CAG GCC CTG AAC TTC AAC AGC GAG ACC GTG CCC CAG AAG

20

TCC TCC CTT GAA GAA CCG GAT TTT TAT AAA ACT AAA ATC AAG CTC TGC ATA CTT CAT AGC AGC CTG GAG GAG CCC GAC TTC TAC AAG ACC AAG ATC AAG CTG TGC ATC CTG CAC AGC AGC CTG GAG GAG CCC GAC TTC TAC AAG ACC AAG ATC AAG CTG TGC ATC CTG CAC

GCT TTC AGA ATT CGG GCA GTG ACT ATT GAC AGA GTG ACG AGC TAT CTG AAT GCT TCC TAA GCC TTC CGC ATC CGC GCC GTG ACC ATC GAC CGC GTG ACC AGC TAC CTG AAC GCC ACC TGA GCC TTC CGC ATC CGG GCC GTG ACC ATC GAC CGC GTG ACC AGC TAC CTG AAC GCC ACG TGA

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Additional Optimized Sequences Coding For IL-12 p35 Subunit (Second Line = SEQ ID NO:24)

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Met Cys Pro Ala Arg Ser Leu Leu Leu Val Ala Thr Leu Val Leu Leu Asp His Leu Ser ATG TGY CCN GCN MGN WSN YTN YTN GTN GCN ACN YTN GTN YTN YTN GAY CAY YTN WSN --- --- --- --- --- --- --- --- --- --- --- --- --- ---

30

ATG TGT CCT GCT CGT TCT TTA TTA GTT GCT ACT TTA GTT TTA GAT CAT TTA TCT TGC CCC GCC CGC TCC TTG TTG GTC GCC ACC TTG GTC TTG TTG GAC CAC TTG TCC CCA GCA CGA TCA CTT CTT GTA GCA ACA CTT GTA CTT CTT CTT TCA CCG GCG CGG TCG CTC CTC GTG GCG ACG CTC GTG CTC CTC CTC TCG

						AC	A AG	т ст	ra ci	ra c	TA			۲.	TA	<u></u>	TA 01				
							G AG								TG		ra ci				TA AGT
														·	10	C.	'G CI	.'G		Cī	rg Agc
												30									
5	Le	u A	la .	Arg	As	n Le	u Pr	o Va	l Al	a Tì	ır Pı	0 A:	sp P	ro Gi	lv Me	t Ph	e Pr	o C		** :	40 s His
	YT	'N G	CN I	MGN	AA	Y YT	и сс	N GT	N GC	N AC	N CO	ON G	Y CO	ON GO	SN AT	G TT	Y (C	осу N тс	o re	u 111	Y CAY
																				N CA	Y CAY
	TT	A GO	T (	CGT	AA'	r tt.	A CC	r GT	T GC	T AC	T CC	T G	AT CO	T GG	T AT	G TT	T CC	т тс	т тт:		T CAT
	TT	G GC	c c	CGC	AA	C TT	G CC	GT	c GC	C AC	c cc	C G	C CC	C GG	iC	TT	c cc	c TG	. TT	- CA	C CAC
10	CT	T GC	:A (	CGA		CT.	r cci	A GT	A GC	A AC	A CC	Α	cc	A GG	A		CC		CT		C CAC
	CT	C GC	G	CGG	•	CT	ccc	GT	G GC	G AC	G CC	G	CC	G GG	G				CTO		
	CT	A	P	AGA		CT													CTA		
	CT	G	A	AGG		CTC	;												CTG		
15											5										60
	Sei	G1:	n A	sn	Leu	Leu	Arg	Ala	Va]	Sei	. Ası	n Me	t Le	u Glı	ı Lys	Ala	Arg	Gln	Thr	Leu	Glu
	WSN	CA	R A	ΑY	YTN	YTN	MGN	GCN	GTN	WS1	AA I	( AT	G YTI	N CAI	R AAR	GCN	MGN	CAR	ACN	YTN	GAR
											·	·									
20	TCI	CAL	AA	AT	TTA	TTA	CGT	GCT	GTT	TCI	' AA1	ATO	TT!	A CAA	AAA	GCT	CGT	CAA	ACT	TTA	GAA
20	TCC	CAC	i Az	AC '	TTG	TTG	CGC	GCC	GTC	TCC	AAC	:	TTO	CAG	AAG	GCC	CGC	CAG	ACC	TTG	GAG
	TCA TCG						CGA									GCA			ACA		
	AGT			,	CTC	CTC	CGG	GCG	GTG	TCG			CTC	;		GCG	CGG		ACG	CTC	
	AGC						AGA						CTA				AGA			CTA	
25	AGC			•	JIG	CTG	AGG			AGC			CTG				AGG			СТG	
	Phe	Tvr	Pr	.o. (	`ve	Th.∽	c	C1	<b>a</b> 1		70										80
	TTY	TAY	CC	יינו	'GY	ACN ACN	nen Sel	GIU	GIU	lle	Asp	His	Glu	Asp	Ile	Thr	Lys	qeA	Lys	Thr	Ser
								GAR	GAK	ATH	GAY				Атн						
30	TTT	TAT																	,		
	TTC	TAC	CC	 Ст	GC .	ACC	TCC	CAC	CAA	ATT	GAT	CAT	GAA	GAT	ATT	ACT	AAA	GAT	AAA .	ACT	TCT
			CC	 А		ACA		JAG			GAC	CAC	GAG		ATC		AAG	GAC	AAG .	ACC	TCC
			CCC			ACG				ATA					ATA	ACA			i	ACA	TCA
							agt									ACG			i	ACG	TCG
35							AGC														AGT
						•															AGC

										90										100
	Thr '	Val	Glu	Ala	Cys	Leu	Pro	Leu	Glu	Leu	Thr	Lys	Asn	Glu	Ser	Cys	Leu	Asn	Ser	Arg
	ACN	GTN	GAR	GCN	TGY	YTN	CCN	YTN	GAR	YTN	ACN	AAR	AAY	GAR	WSN	TGY	YTN	AAY	WSN	MGN
5	ACT	GTT	GAA	GCT	TGT	TTA	ССТ	TTA	GAA	TTA	ACT	AAA	AAT	GAA	TCT	TGT	TTA	AAT	TCT	CGT
	ACC	GTC	GAG	GCC	TGC	TTG	ccc	TTG	GAG	TTG	ACC	AAG	AAC	GAG	TCC	TGC	TTG	AAC	TCC	CGC
	ACA	GTA		GCA		CTT	CCA	CTT		CTT	ACA				TCA		CTT		TCA	CGA
	ACG	GTG		GCG		CTC	CCG	CTC		CTC	ACG				TCG		CTC		TCG	CGG
						СТА		CTA		CTA					AGT		CTA		AGT	AGA
10						CTG		CTG		CTG					AGC		CTG		AGC	AGG
										110										120
	Glu	Thr	Ser	Phe	Ile	Thr	Asn	Gly	Ser	Cys	Leu	Ala	Ser	Arg	Lys	Thr	Ser	Phe	Met	Met
	GAR	ACN	WSN	TTY	АТН	ACN	AAY	GGN	WSN	TGY	YTN	GCN	WSN	MGN	AAR	ACN	WSN	TTY	ATG	ATG
15																	<b>-</b>		<b></b>	
															AAA					ATG
	GAG	ACC	TCC	TTC	ATC	ACC	AAC	GGC	TCC	TGC	TTG	GCC	TCC	CGC	AAG	ACC	TCC	TTC		
		ACA	TCA		ATA	ACA		GGA	TCA		CTT	GCA	TCA	CGA		ACA	TCĀ			
•		ACG	TCG			ACG		GGG	TCG		CTC	GCG	TCG	CGG		ACG	TCG			
20			AGT																	
			AGC						AGC		CTG		AGC	AGG			AGC			
										130										140
																				Thr
25	GCN	YTN	TGY										ATG	TAY	CAR	GTN	GAR	TTY	AAR	ACN
					<b>-</b>															
																				ACT
	GCC	TTG	TGC	TTG	TCC	TCC	ATC	TAC	GAG	GAC			;	TAC	CAG			TTC	. AAG	ACC
	GCA	CTT	•		TCA						CTI					GTA				ACA
30	GCG	CTC	:	CTC							CTC					GTG	,			ACG
		CTA	١.	CTF	AGT	AGT	•				CTA									
		CTC	;	CTC	AGC	: AGC	:				CTG	;								

										15										160
	Met	As	n Al	a Ly	s Le	u Lei	ı Me	t As	p Pr	o Ly	s Ar	g G1	n Il	e Ph	e Le	u As	p G1	n A:	sn Me	et Leu
	ATG	AA'	Y GC	AA N	R YT	N YTI	TA P	G GA	Y CC	н аа	R MG	N CA	R AT	н тт	Y YT	N GA	Y CP	LR AZ	AY AT	G YTN
				- <del>-</del> -				- <b>-</b>					<del>-</del>				- <u>-</u> -			
5	ATG	AA:	r GC	C AA	A TT	A TT	ATC	GA.	L CC.	T AA	A CG	т са	а ат	т тт	T TT.	A GA	T CA	A A	ra T	G TTA
		AAC	GC	AA(	3 <b>T</b> T(	G TTC	;	GAG	CCC	C AA	G CG	C CA	G AT	СТТ	C TT	G GA	C CA	G A,	ıc	TTG
			GC			г сті			CC		CG.		AT.		CT					CTT
			GCC	3	CTO	CTC	;		CC	3	CG	G			CT	3				CTC
					CTA	A CTA					AG	A			CT	A.				CTA
10					CTC	CTG					AG	3			СТО	3				CTG
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										170	)									180
	Ala	Val	Ile	Asp	Glu	Leu	Met	Gln	Ala	Leu	Asr	n Phe	e Asr	ı Seı	Glu	Thr	: Val	l Pr	o Gli	ı Lys
	GCN	GTN	АТН	GAY	GAR	YTN	ATG	CAR	GCN	YTN	AA	TTY	. AAY	. WSI	I GAR	ACN	GTN	I CCI	N CAI	AAR
15																				
	GCT	GTT	ATT	GAT	GAA	TTA	ATG	CAA	GCT	TTA	AAT	TTT	' AAT	TCI	' GAA	ACT	GTT	' CC'	ר רמי	AAA
	GCC	GTC	ATC	GAC	GAG	TTG		CAG	GCC	TTG	AAC	TTC	AAC	TCC	GAG	ACC	GTC		י כאס	AAG
	GCA	GTA	ATA			CTT			GCA							ACA				, ANG
	GCG	GTG				CTC			GCG	CTC										
20						CTA				CTA				AGT					•	
						CTG				CTG				AGC						
										190										200
	Ser S	Ser	Leu	Glu	Glu	Pro	Asp	Phe	Tyr	Lys	Thr	Lys	Ile	Lys	Leu	Cvs	Tle	Len	Lau	
25	WSN W	<b>N</b> SN	YTN	GAR	GAR	CCN	GAY	TTY	TAY	AAR	ACN	AAR	ATH	AAR	YTN	TGY	АТН	YTN	YTM	UIS
	TCT 1	CT	TTA	GAA	GAA	CCT	GAT	TTT	TAT	AAA	ACT	AAA	АТТ	AAA	TTA	TGT	АТТ	ጥፐል	ጥጥል	CAT
	TCC T	cc	TTG	GAG	GAG	ccc (	GAC '	TTC	TAC	AAG	ACC	AAG	ATC	AAG	TTG	TGC	ልጥር	יייים.	ጥጥር	CNC
	TCA T	CA	CTT			CCA					ACA		ATA		CTT					CAC
30	TCG T	.ce	CTC			CCG					ACG				CTC			CTC		
	AGT A	GT	CTA												CTA			CTA		
	AGC A	GC (	CTG												CTG			CTG		
																		CIG	CTG	

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220 210 Ala Phe Arg Ile Arg Ala Val Thr Ile Asp Arg Val Thr Ser Tyr Leu Asn Ala Ser \*\*\* GCN TTY MGN ATH MGN GCN GTN ACN ATH GAY MGN GTN ACN WSN TAY YTN AAY GCN WSN TRR \_\_\_ \_\_ \_\_ \_\_ \_\_ \_\_ \_\_ \_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ \_\_\_ GCT TTT CGT ATT CGT GCT GTT ACT ATT GAT CGT GTT ACT TCT TAT TTA AAT GCT TCT TAA 5 GCC TTC CGC ATC CGC GCC GTC ACC ATC GAC CGC GTC ACC TCC TAC TTG AAC GCC TCC TAG GCA TCA TGA CTT CGA GTA ACA TCA CGA ATA CGA GCA GTA ACA ATA GCA GCG TCG CTC CGG GCG GTG ACG CGG GTG ACG TCG CGG GCG AGT CTA AGT AGA AGA AGA AGC CTG AGG AGC 10 AGG AGG Sequences Encoding Human IL-12 p40 First line = natural sequence (SEQ ID NO. 2) Second line = all codons optimized (SEQ ID NO. 3) Third line = all codons optimized except when same close/abundant (changes acids were too between 15 second and third lines bolded) (SEQ ID NO. 4) ATG TGT CAC CAG CAG TTG GTC ATC TCT TGG TTT TCC CTG GTT TTT CTG GCA TCT CCC CTC ATG TGC CAC CAG CAG CTG GTG ATC AGC TGG TTC AGC CTG GTG TTC CTG GCC AGC CCC CTG ATG TGC CAC CAG CAG CTG GTG ATC AGC TGG TTC TCC CTG GTG TTT CTG GCC AGC CCC CTC 20 GTG GCC ATA TGG GAA CTG AAG AAA GAT GTT TAT GTC GTA GAA TTG GAT TGG TAT CCG GAT GTG GCC ATC TGG GAG CTG AAG AAG GAC GTG TAC GTG GTG GAG CTG GAC TGG TAC CCC GAC GTG GCC ATC TGG GAG CTG AAG AAA GAC GTG TAC GTG GTC GAG CTG GAC TGG TAC CCC GAC GCC CCT GGA GAA ATG GTG GTC CTC ACC TGT GAC ACC CCT GAA GAA GAT GGT ATC ACC TGG 25 GCC CCC GGC GAG ATG GTG GTG CTG ACC TGC GAC ACC CCC GAG GAG GAC GGC ATC ACC TGG GCC CCC GGC GAG ATG GTG GTC CTG ACC TGC GAC ACC CCC GAG GAA GAC GGC ATC ACC TGG ACC TTG GAC CAG AGC AGT GAG GTC TTA GGC TCT GGC AAA ACC CTG ACC ATC CAA GTC AAA ACC CTG GAC CAG AGC AGC GAG GTG CTG GGC AGC GGC AAG ACC CTG ACC ATC CAG GTG AAG 30 ACC CTG GAC CAG AGC AGT GAG GTG CTG GGC TCC GGC AAG ACC CTG ACC ATC CAG GTG AAG GAG TTT GGA GAT GCT GGC CAG TAC ACC TGT CAC AAA GGA GGC GAG GTT CTA AGC CAT TCG

GAG TTC GGC GAC GCC GGC CAG TAC ACC TGC CAC AAG GGC GAG GTG CTG AGC CAC AGC GAG TTC GGC GAC GCC GGC CAG TAC ACC TGC CAC AAG GGA GGC GAG GTG CTG AGC CAC TCC

CTC CTG CTG CTG CAC AAA AAG GAA GAT GGA ATT TGG TCC ACT GAT ATT TTA AAG GAC CAG
CTG CTG CTG CTG CAC AAG AAG GAG GAC GGC ATC TGG AGC ACC GAC ATC CTG AAG GAC CAG
CTC CTG CTG CTC CAC AAA AAG GAG GAC GGC ATC TGG AGC ACC GAC ATC CTG AAG GAC CAG

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ACC TGC TGG TGG CTG ACG ACA ATC AGT ACT GAT TTG ACA TTC AGT GTC AAA AGC AGC AGA
ACC TGC TGG TGG CTG ACC ACC ATC AGC ACC GAC CTG ACC TTC AGG GTG AAG AGC AGC AGG
ACC TGC TGG TGG CTG ACC ACG ATC AGC ACC GAC CTG ACC TTC AGT GTG AAG AGC AGC AGG

GGC TCT TCT GAC CCC CAA GGG GTG ACG TGC GGA GCT GCT ACA CTC TCT GCA GAG AGA GTC

15 GGC AGC AGC GAC CCC CAG GGC GTG ACC TGC GGC GCC GCC ACC CTG AGC GCC GAG CGC GTG

GGC TCC AGC GAC CCC CAG GGC GTG ACC TGC GGC GCC ACC CTG AGC GCC GAG CGC GTG

AGA GGG GAC AAC AAG GAG TAT GAG TAC TCA GTG GAG TGC CAG GAG GAC AGT GCC TGC CCA CGC GGC GAC AAC AAG GAG TAC GAG TAC AGC GTG GAG TGC CAG GAG GAC AGC GCC TGC CCC CGC GGC GAC AAC AAG GAG TAC GAG TAC AGC GTG GAG TGC CAG GAA GAC TCC GCC TGC CCC

GCT GCT GAG GAG AGT CTG CCC ATT GAG GTC ATG GTG GAT GCC GTT CAC AAG CTC AAG TAT
GCC GCC GAG GAG AGC CTG CCC ATC GAG GTG ATG GTG GAC GCC GTC CAC AAG CTG AAG TAC
GCC GCT GAG GAG AGC CTG CCC ATC GAG GTG ATG GTG GAC GCC GTT CAC AAG CTG AAG TAC

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5 CGC AAA AAT GCC AGC ATT AGC GTG CGG GCC CAG GAC CGC TAC TAT AGC TCA TCT TGG AGC CGC AAG AAC GCC AGC ATC AGC GTG CGC GCC CAG GAC CGC TAC TAC AGC AGC AGC AGC AGC CGC AAG AAC GCC AGC ATC AGC GTG CGC CAG GAC CGC TAC TAT AGC TCC TCT TGG AGC

GAA TGG GCA TCT GTG CCC TGC AGT TAG

10 GAG TGG GCC AGC GTG CCC TGC AGC TAG

GAG TGG GCC AGC GTG CCC TGC TCC TAG

### Additional Optimized Sequences Coding For IL-12 p40 Subunit (Second Line = SEQ ID NO:25)

10 20 Met Cys His Gln Gln Leu Val Ile Ser Trp Phe Ser Leu Val Phe Leu Ala Ser Pro Leu 15 ATG TGY CAY CAR CAR YTN GTN ATH WSN TGG TTY WSN YTN GTN TTY YTN GCN WSN CCN YTN ATG TGT CAT CAA CAA TTA GTT ATT TCT TGG TTT TCT TTA GTT TTT TTA GCT TCT CCT TTA TGC CAC CAG CAG TTG GTC ATC TCC TTC TCC TTG GTC TTC TTG GCC TCC CCC TTG 20 TCA CTT GTA CTT GCA TCA CCA CTT CTT GTA ATA TCA CTC GTG TCG CTC GTG CTC GCG TCG CCG CTC TCG AGT CTA CTA AGT CTA AGT CTA CTG CTG AGC AGC CTG AGC CTG 25 30 40 Val Ala Ile Trp Glu Leu Lys Lys Asp Val Tyr Val Val Glu Leu Asp Trp Tyr Pro Asp GTN GCN ATH TGG GAR YTN AAR AAR GAY GTN TAY GTN GTN GAR YTN GAY TGG TAY CCN GAY GTT GCT ATT TGG GAA TTA AAA AAA GAT GTT TAT GTT GAA TTA GAT TGG TAT CCT GAT 30 GTC GCC ATC GAG TTG AAG AAG GAC GTC TAC GTC GTC GAG TTG GAC TAC CCC GAC GTA GCA ATA CTT GTA GTA GTA CTT CCA GTG GCG CTC GTG GTG GTG CTC CCG CTA CTA CTG CTG

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										50	ı									60
	Ala	Pro	Gly	Glu	Met	Val	Val	Leu	Thr	Cys	Asp	Thr	Pro	Glu	Glu	Asp	Gly	Ile	Thr	Trp
	GCN	CCN	GGN	GAR	ATG	GTN	GTN	YTN	ACN	TGY	GAY	ACN	CCN	GAR	GAR	GAY	GGN	АТН	ACN	TGG
											<del>-</del>									
5	GCT	CCT	GGT	GAA	ATG	GTT	GTT	TTA	ACT	TGT	GAT	ACT	CCT	GAA	GAA	GAT	GGT	ATT	ACT	TGG
	GCC	ccc	GGC	GAG		GTC	GTC	TTG	ACC	TGC	GAC	ACC	ccc	GAG	GAG	GAC	GGC	ATC	ACC	
	GCA	CCA	GGA			GTA	GTA	CTT	ACA			ACA	CCA				GGA	ATA	ACA	
	GCG	CCG	GGG			GTG	GTG	CTC	ACG			ACG	CCG				GGG		ACG	
								CTA												
10								CTG												
															-					
										70										80
	Thr	Leu	Asp	Gln	Ser	Ser	Glu	Val	Leu	Gly	Ser	Gly	Lys	Thr	Leu	Thr	Ile	Gln	Val	Lys
			GAY																	
15																				
	ACT	TTA	GAT	CAA	TCT	TCT	GAA	GTT	TTA	GGT	TCT	GGT	AAA	ACT	TTA	ACT	ATT	CAA	GTT	AAA
			GAC																	
	ACA					TCA									CTT				GTA	
	ACG	CTC			TCG	TCG									CTC				GTG	
20		CTA			AGT	AGT			CTA		AGT				CTA					
		CTG			AGC	AGC			CTG		AGC				CTG.					
										90										100
	Glu	Phe	Gly	Asp	Ala	Gly	Gln	Tyr	Thr	Cys	His	Lys	Gly	Gly	Glu	Val	Leu	Ser	His	Ser
25			GGN																	
	GAA	TTT	GGT	GAT	GCT	GGT	CAA	TAT	ACT	TGT	CAT	AAA	GGT	GGT	GAA	GTT	TTA	TCT	CAT	тст
			GGC																	
			GGA		GCA				ACA						(					TCA
30			GGG		GCG	GGG			ACG						(					
														-			CTA .			AGT
																	CTG .			AGC

BRIEDOCIO AMO MAZOZBANIA.

										110										120
	Leu	Leu	Leu	Leu	His	Lys	Lys	Glu	Asp	Gly	Ile	Trp	Ser	Thr	Asp	Ile	Leu	Lys	Asp	Gln
-	YTN	YTN	YTN	YTN	CAY	AAR	AAR	GAR	GAY	GGN	ATH	TGG	WSN	ACN	GAY	ATH	YTN	AAR	GAY	CAR
5	TTA	TTA	TTA	TTA	CAT	AAA	AAA	GAA	GAT	GGT	ATT	TGG	TCT	ACT	GAT	ATT	TTA	AAA	GAT	CAA
	TTG	TTG	TTG	TTG	CAC	AAG	AAG	GAG	GAC	GGC	ATC		TCC	ACC	GAC	ATC	TTG	AAG	GAC	CAG
	CTT	CTT	CTT	CTT						GGA <sub>.</sub>	ATA		TCA	ACA		ATA	CTT			
	CTC	CTC	CTC	CTC						GGG			TCG	ACG			CTC			
	CTA	CTA	CTA	CTA									AGT				CTA			
10	CTG	CTG	CTG	CTG									AGC				CTG			
															ā					
										130										140
	Lys	Glu	Pro	Lys	Asn	Lys	Thr	Phe	Leu	Arg	Суѕ	Glu	Ala	Lys	Asn	Tyr	Ser	Gly	Arg	Phe
	AAR	GAR	CCN	AAR	AAY	AAR	ACN	TTY	YTN	MGN	TGY	GAR	GCN	AAR	AAY	TAY	WSN	GGN	MGN	TTY
15																				
	AAA	GAA	CCT	AAA	AAT	AAA	ACT	TTT	TTA	CGT	TGT	GAA	GCT	AAA	AAT	TAT	TCT	GGT	CGT	TTT
	AAG	GAG	CCC	AAG	AAC	AAG	ACC	TTC	TTG	CGC	TGC	GAG	GCC	AAG	AAC	TAC	TCC	GGC	CGC	TT,C
			CCA				ACA		CTT	CGA			GCA				TCA	GGA	CGA	
			CCG				ACG		CTC	CGG			GCG				TCG	GGG	CGG	
20									CTA	AGA							AGT		AGA	
									CTG	AGG							AGC		AGG	
•																				
										150										160
		-	-	-					•		_			Phe			_			_
25	ACN	TGY	TGG	TGG	YTN	ACN	ACN					•		TTY	•				-	
				TGG										TTT						
		TGC												TTC						
2.0	ACA											CTT			TCA					
30	ACG					ACG						CTC	ACG		TCG			TCG		
					CTA							CTA			AGT					
					CTG				AGC			CTG			AGC			AGC	AGC	AGG

48

										170										180
	Gly	Ser	Ser	Asp	Pro	Gln	Gly	Val	Thr	Cys	Gly	Ala	Ala	Thr	Leu	Ser	Ala	Glu	Arg	Va]
	GGN	WSN	WSN	GAY	CCN	CAR	GGN	GTN	ACN	TGY	GGN	GCN	GCN	ACN	YTN	WSN	GCN	GAR	MGN	GTN
																				- <b>-</b> -
5	GGT	TCT	TCT	GAT	CCT	CAA	GGT	GTT	ACT	TGT	GGT	GCT	GCT	ACT	TTA	TCT	GCT	GAA	CGT	GTI
	GGC	TCC	TCC	GAC	ccc	CAG	GGC	GTC	ACC	TGC	GGC	GCC	GCC	ACC	TTG	TCC	GCC	GAG	CGC	GTC
	GGA	TCA	TCA		CCA		GGA	GTA	ACA		GGA	GCA	GCA	ACA	CTT	TCA	GCA		CGA	GTA
	GGG	TCG	TCG		CCG		GGG	GTG	ACG		GGG	GCG	GCG	ACG	CTC	TCG	GCG		CGG	GTG
		AGT	AGT												CTA	AGT			AGA	
10		AGC	AGC												CTG	AGC			AGG	
															z		•			
										190										200
	Arg	Gly	Asp	Asn	Lys	Glu	Tyr	Glu	Tyr	Ser	Val	Glu	Cys	Gln	Glu	Asp	Ser	Ala	Cys	Pro
	MGN	GGN	GAY	AAY	AAR	GAR	TAY	GAR	TAY	WSN	GTN	GAR	TGY	CAR	GAR	GAY	WSN	GCN	TGY	CCN
15							<b>-</b>													
	CGT	GGT	GAT	AAT	AAA	GAA	TAT	GAA	TAT	TCT	GTT	GAA	TGT	CAA	GAA	GAT	TCT	GCT	TGT	ССТ
	CGC	GGC	GAC	AAC	AAG	GAG	TAC	GAG	TAC	TCC	GTC	GAG	TGC	CAG	GAG	GAC	TCC	GCC	TGC	ccc
	CGA	GGA								TCA	GTA						TCA	GCA		CCA
	CGG	GGG								TCG	GTG						TCG	GCG		CCG
20	AGA									AGT							AGT			
	AGG									AGC							AGC			
										210										220
	Ala	Ala	Glu	Glu	Ser	Leu	Pro	Ile	Glu	Val	Met	Val	Asp	Ala	Val	His	Lys	Leu	Lys	Tyr
25												GTN								
	GCT	GCT	GAA	GAA	TCT	TTA	ССТ	ATT	GAA	GTT	ATG	GTT	GAT	GCT	GTT	CAT	AAA	ATT	AAA	TAT
	GCC	GCC	GAG	GAG	TCC	TTG	ccc	ATC	GAG	GTC		GTC	GAC	GCC	GTC	CAC	AAG	TTG	AAG	TAC
	GCA	GCA			TCA	CTT	CCA	ATA		GTA		GTA		GCA	GTA			СТТ		
30	GCG	GCG			TCG	СТС	CCG			GTG		GTG		GCG	GTG			CTC		
					AGT	CTA												СТА		
					AGC	CTG												CTG		

BUSINGID JING - MATETRAS IAS

										230										240
	Glu	Asn	Tyr	Thr	Ser	Ser	Phe	Phe	Ile	Arg	Asp	Ile	Ile	Lys	Pro	qeA	Pro	Pro	Lys	Asn
	GAR	AAY	TAY	ACN	WSN	WSN	TTY	TTY	нта	MGN	GAY	ATH	ATH	AAR	CCN	GAY	CCN	CCN	AAR	AAY
5	GAA	TAA	TAT	ACT	TCT	TCT	TTT	TTT	ATT	CGT	GAT	ATT	ATT	AAA	CCT	GAT	ССТ	CCT	AAA	TAA
	GAG	AAC	TAC	ACC	TCC	TCC	TTC	TTC	ATC	CGC	GAC	ATC	ATC	AAG	ccc	GAC	ccc	ccc	AAG	AAC
				ACA	TCA	TCA			ATA	CGA		ATA	ATA		CCA		CCA	CCA		
				ACG	TCG	TCG				CGG					CCG		CCG	CCG		
					AGT	AGT				AGA										
10					AGC	AGC				AGG										
															-					
•										250										260
	Leu	Gln	Leu	Lys	Pro	Leu	Lys	Asn	Ser	Arg	Gln	Val	Glu	Val	Ser	Trp	Glu	Tyr	Pro	Asp
	YTN	CAR	YTN	AAR	CCN	YTN	AAR	AAY	WSN	MGN	CAR	GTN	GAR	GTN	WSN	TGG	GAR	TAY	CCN	GAY
15																				<u>-</u>
•	TTA	CAA	TTA	AAA	CCT	TTA	AAA	AAT	TCT	CGT	CAA	GTT	GAA	GTT	TCT	TGG	GAA	TAT	CCT	GAT
	TTG	CAG	TTG	AAG	ccc	TTG	AAG	AAC	TCC	CGC	CAG	GTC	GAG	GTC	TCC		GAG	TAC	ccc	GAC
	CTT		CTT		CCA	CTT			TCA	CGA		GTA		GTA	TCA				CCA	93
	CTC		CTC		CCG	CTC			TCG	CGG		GTG		GTG	TCG				CCG	
20	CTA		CTA			CTA			AGT	AGA					AGT					
	CTG		CTG			CTG			AGC	AGG					AGC					
										270										280
	Thr	Trp	Ser	Thr	Pro	His	Ser	Tyr	Phe	Ser	Leu	Thr	Phe	Cys	Val	Gln	Val	Gln	Gly	Lys
25	ACN	TGG	WSN	ACN	CCN	CAY	WSN	TAY	TTY	WSN	YTN	ACN	TTY	TGY	GTN	CAR	GTN	CAR	GGN	AAR
	ACT	TGG	TCT	ACT	CCT	CAT	TCT	TAT	TTT	TCT	TTA	ACT	TTT	TGT	GTT	CAA	GTT	CAA	GGT	AAA
	ACC		TCC	ACC	ccc	CAC	TCC	TAC	TTC	TCC	TTG	ACC	TTC	TGC	GTC	CAG	GTC	CAG	GGC	AAG
	ACA		TCA	ACA	CCA		TCA			TCA	CTT	ACA			GTA		GTA		GGA	
30	ACG		TCG	ACG	CCG		TCG			TCG	CTC	ACG			GTG		GTG		GGG	
			AGT				AGT			AGT	CTA									
			AGC				AGC			AGC	CTG									

290 300 Ser Lys Arg Glu Lys Lys Asp Arg Val Phe Thr Asp Lys Thr Ser Ala Thr Val Ile Cys WSN AAR MGN GAR AAR GAY MGN GTN TTY ACN GAY AAR ACN WSN GCN ACN GTN ATH TGY 5 TCT AAA CGT GAA AAA AAA GAT CGT GTT TTT ACT GAT AAA ACT TCT GCT ACT GTT ATT TGT TCC AAG CGC GAG AAG AAG GAC CGC GTC TTC ACC GAC AAG ACC TCC GCC ACC GTC ATC TGC TCA CGA CGA GTA ACA ACA TCA GCA ACA GTA ATA TCG CGG CGG GTG ACG ACG TCG GCG ACG GTG AGT AGA AGA AGT 10 AGC AGG AGG AGC 310 320 Arg Lys Asn Ala Ser Ile Ser Val Arg Ala Gln Asp Arg Tyr Tyr Ser Ser Ser Trp Ser MGN AAR AAY GCN WSN ATH WSN GTN MGN GCN CAR GAY MGN TAY TAY WSN WSN WSN TGG WSN 15 --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---CGT AAA AAT GCT TCT ATT TCT GTT CGT GCT CAA GAT CGT TAT TAT TCT TCT TGG TCT CGC AAG AAC GCC TCC ATC TCC GTC CGC GCC CAG GAC CGC TAC TAC TCC TCC CGA GCA TCA ATA TCA GTA CGA GCA CGA TCA TCA TCA TCA CGG GCG TCG TCG GTG CGG GCG CGG TCG TCG TCG TCG 20 AGA AGT AGT AGA AGA AGT AGT AGT AGT AGG AGC AGC AGG AGG AGC AGC AGC AGC Glu Trp Ala Ser Val Pro Cys Ser \*\*\* 25 GAR TGG GCN WSN GTN CCN TGY WSN TRR --- --- --- --- --- --- ---GAA TGG GCT TCT GTT CCT TGT TCT TAA GAG GCC TCC GTC CCC TGC TCC TAG GCA TCA GTA CCA TCA TGA 30 GCG TCG GTG CCG TCG AGT AGT

DESCRIPTION OF THE PARTY IN

AGC

AGC

	Wi]	ld T	уре	Se	que	nce	Co	din	g F	or	Int	erf	ero	n A	lph	a			
			9	)		18	1		27			36	;		45	•		54	
	5'	ATG	GCC	TTG	ACC	TTT	GCT	TTA	CTG	GTG	GCC	CTC	CTG	GTG	CTC	AGC	TGC	AAG	TCA
																	<b></b>		
5		M	A	L	T	F	A	L	L	v	A	L	L	v	L	S	С.	ĸ	S
				53												9			
				TCT							CAA	ACC	CAC	AGC	CTG	GGT	AGC	AGG	AGG
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10		S	С	s.	V	G	С	D	L	P	Q	Т	н	5			5	R	K
				17		1.	3.6		1 7	15		1.4	14		19			16	52
		x c c		ATG															
15		т	L	м	L	L	A	Q	М	R	R	I	s	L	F	S	C	L	ĸ
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			1	71		1	80		18	39		1.9	98		20	07		2:	16 .
		GAC	AGA	CAT	GAC	TTT	GGA	TTT	ccc	CAG	GAG	GAG	TTT	GGC	AAC	CAG	TTC	CAA	AAG
20		D	R	Н	D	F	G	F	P	Q	E	E	F	G	N	Q	F	Q	K
				25															
		GCT	GAA	ACC	ATC												AAT	CTC	TTC
25		A	E	T	I	P	V	L	Н	E	M	Ι	Q	Q	I	F	N	L	F
			_	79		_						,	0.0		2	15		3	24
				79 AAG															
		AGC	ACA	AAG	GAC	TCA	TCT	GCT	GC1	166	GAI	GAG							
30		s	<b></b> -	 к	D			A	Δ.	W	ם	E	т	L	L	D	к	F	Y
30		S		K	U		_	••	••	•	_	_							
			3	33		3	42		3	51		3	60		3	69		3	78
		ACT		CTC	TAC			CTG						TGT	GTG	ATA	CAG	GGG	GTG
35		Ŧ	E	L	Y	Q	Q	L	N	D	L	E	A	С	V	I	Q	G	v

			3	96		4	05		4	14		4	23		4	32		
	GGG	GTG	ACA	GAG	ACT	ccc	CTG	ATG	AAG	GAG	GAC	TCC	ATT	CTG	GCT	GTG		AAA
				<del>-</del>														
_	G	v	T	E	T	₽	L	M	ĸ	E	D	s	I	L	A	v	R	ĸ
5																		
			41								4							86
	TAC	TTC	CAA	AGA	ATC	ACT	CTC	TAT	CTG	AAA	GAG	AAG	AAA	TAC	AGC	CCT	TGT	GCC
10	Y	F	Q	R	I	T	L	Y	L	K	E	ĸ	ĸ	Y	s	P	С	A
10																		
			95		50						52							
	TGG	GAG	GTT	GTC	AGA	GCA	GAA	ATC	ATG	AGA	TCT	TTT	TCT	TTG	TCA	ACA	AAC	TTG
										<b>-</b>								
15	W	E	V	V	R	A	E	I	M	R	s	F	S	L	s	T	N	L
15																		
					•			56										
	CAA	GAA	AGT	TTA	AGA	AGT .	AAG (	GAA	TGA	3'								
	Q E S L			R	S	ĸ	E	*										

# 20 Interferon Alpha Coding Sequence with All Codons Optimized (SEQ ID NO:11)

GCC GTG CGG AAG TAC TTC CAG CGG ATC ACC CTG TAC CTG AAG GAG AAG AAG TAC TCC CCC

TGC GCC TGG GAG GTG GTG CGG GCC GAG ATC ATG CGG AGC TTC AGC CTG AGC ACC AAC CTG

CAG GAG AGC CTG CGG AGC AAG GAG TGA

## Additional/Semi Optimized Sequence Coding For Interferon Alpha (Second Line = SEQ ID NO:12)

10										10	)									20
	MET	ALA	LEU	THR	PHE	ALA	LEU	LEU	VAL	ALA	LEU	LEU	VAL	LEU	SER	CYS	LYS	SER	SER	CYS
	ATG	GCN	YTN	ACN	TTY	GCN	YTN	YTN	GTN	GCN	YTN	YTN	GTN	YTN	WSN	TGY	AAR	WSN	WSN	TGY
													<b>-</b>						<del>-</del>	
	ATG			ACT																
15		GCC	TTG	ACC	TTC	GCC	TTG	TTG	GTC	GCC	TTG	TTG	GTC	TTG	TCC	TGC	AAG	TCC	TCC	TGC
		GCA	CTT	ACA		GCA	CTT	CTT	GTA	GCA	CTT	CTT	GTA	CTT	TCA			TCA	TCA	
		GCG	CTC	ACG		GCG	CTC	CTC	GTG	GCG	CTC	CTC	GTG	CTC	TCG			TCG	TCG	
			CTA				CTA	CTA			CTA	CTA		CTA	AGT			AGT	AGT	
			CTG				CTG	CTG			CTG	CTG		CTG	AGC			AGC	AGC	
20																				
										30										40
	SER	VAL	GLY	CYS	ASP	LEU	PRO	GLN	THR	HIS	SER	LEU	GLY	SER	ARG	ARG	THR	LEU	MET	LEU
	WSN	GTN	GGN	TGY	GAY	YTN	CCN	CAR	ACN	CAY	WSN	YTN	GGN	WSN	MGN	MGN	ACN	YTN ·	ATG	YTN
25	TCT	GTT	GGT	TGT	GAT	TTA	CCT	CAA	ACT	CAT	TCT	TTA	GGT	TCT	CGT	CGT	ACT	TTA	ATG	TTA
	TCC	GTC	GGC	TGC	GAC	TTG	CCC	CAG	ACC	CAC	TCC	TTG	GGC	TCC	CGC	CGC	ACC	TTG		TTG
	TCA	GTA	GGA			CTT	CCA		ACA		TCA	CTT	GGA	TCA	CGA	CGA	ACA	CTT		CTT
	TCG	GTG	GGG	i		CTC	CCG		ACG		тCG	CTC	GGG	TCG	CGG	CGG	ACG	CTC		CTC
	AGT	•				СТА					AGT	CTA		AGT	AGA	AGA		CTA		CTA
30	AGC	:				CTG					AGC	СТG		AGC	AGG	AGG		CTG		CTG

		•																		
										50										60
				N MET																
	YTI	≀ GCI		R ATC														GAY	TTY	GGN
_				- <del>-</del>																
5				A ATO																
				3	CGC	CGC	ATC	TCC	TTG	TTC	TCC	TGC	TTG	AAG	GAC	CGC	CAC	GAC	TTC	GGC
		GC)									TCF		CTT			CGA				GGA
			3		CGG	CGG		TCG	CTC		TCG	;	CTC			CGG	;			GGG
• •	CTA				AGA	AGA		AGT	CTA		AGT	•	CTA			AGA				
10	CTG	i			AGG	AGG		AGC	CTG		AGC	:	CTG			AGG				
															2					
										70										80
				GLU																
	TTY			GAR																
15																				
				GAA																
	TTC			GAG	GAG	TTC	GGC	AAC	CAG	TTC	CAG	AAG	GCC	GAG	ACC	ATC	ccc	GTC	TTG	CAC
		CCA					GGA						GCA		ACA	ATA	CCA	GTA	CTT	
20		CCG					GGG						GCG		ACG		CCG	GTG	CTC	
20																			CTA	
																			CTG	
										90										100
2.5				GLN																
25	GAR	ATG	ATH	CAR	CAR													GCN	TGG	GAY
				CAA																GAT
	GAG			CAG	CAG	ATC	TTC	AAC	TTG	TTC	TCC	ACC	AAG	GAC '	TCC '	TCC	GCC	GCC	1	GAC
20			ATA		i	ATA			CTT		TCA	ACA		•	TCA '	TCA	GCA	GCA		
30									CTC		TCG	ACG		•	rcg :	TCG	GCG	GCG		
									CTA		AGT			i	AGT I	AGT				
									CTG		AGC			2	AGC 1	AGC				

QUICHTONIN SHIP PORTETONE IA-

										110										120
	GLU	THR	LEU	LEU	ASP	LYS	PHE	TYR	THR	GLU	LEU	TYR	GLN	GLN	LEU	ASN	ASP	LEU	GLU	ALA
	GAR	ACN	YTN	YTN	GAY	AAR	TTY	TAY	ACN	GAR	YTN	YAT	CAR	CAR	YTN	AAY	GAY	YTN	GAR	GCN
																<b>-</b>				
5	GAA	ACT	TTA	TTA	GAT	AAA	TTT	TAT	ACT	GAA	TTA	TAT	CAA	CAA	TTA	AAT	GAT	TTA	GAA	GCT
	GAG	ACC	TTG	TTG	GAC	AAG	TTC	TAC	ACC	GAG	TTG	TAC	CAG	CAG	TTG	AAC	GAC	TTG	GAG	GCC
		ACA	CTT	СТТ					ACA		CTT				CTT			CTT		GCA
		ACG	СТС	CTC					ACG		СТС				CTC			CTC		GCG
			CTA	CTA							CTA				CTA			CTA		
10			CTG	CTG						,	CTG				CTG			CTG		
															z					
										130										140
	CYS	VAL	ILE	GLN	GLY	VAL	GLY	VAL	THR	GLU	THR	PRO	LEU	MET	LYS	GLU	ASP	SER	ILE	LEU
•	TGY	GTN	АТН	CAR	GGN	GTN	GGN	GTN	ACN	GAR	ACN	CCN	YTN	ATG	AAR	GAR	GAY	WSN	ĄТН	YTN
15																				
	TGT	GTT	ATT	CAA	GGT	GTT	GGT	GTT	ACT	GAA	ACT	CCT	TTA	ATG	AAA	GAA	GAT	TCT	ATT	TTA
•	TGC	GTC	ATC	CAG	GGC	GTC	GGC	GTC	ACC	GAG	ACC	ccc	TTG		AAG	GAG	GAC	TCC	ATC	TTG
		GTA	ATA		GGA	GTA	GGA	GTA	ACA		ACA	CCA	CTT					TCA	ATA	CTT
		GTG			GGG	GTG	GGG	GTG	ACG		ACG	CCG	CTC					TCG		CTC
20	•												CTA					AGT		CTA
													CTG					AGC		CTG
										150										160
	ALA	VAL	ARG	LYS	TYR	PHE	GLN	ARG	ILE	THR	LEU	TYR	LEU	LYS	GLU	LYS	LYS	TYR	SER	PRO
25	GCN	GTN	MGN	AAR	TAY	TTY	CAR	MGN	АТН	ACN	YTN	TAY	YTN	AAR	GAR	AAR	AAR	TAY	WSN	CCN
•																				
	GCT	GTI	CGT	' AAA	TAT	TTT	CAA	CGT	ATT	ACT	TTA	TAT	TTA	AAA	GAA	AAA	AAA	TAT	TCT	CCT
	GCC	GTC	: CGC	: AAG	TAC	TTC	CAG	CGC	ATC	ACC	TTG	TAC	TTG	AAG	GAG	AAG	AAG	TAC	TCC	ccc
	GCA	GTA	CGA	١				ÇGA	ATA	ACA	CTT		CTT						TCA	CCA
30	GCG	GTG	CGG	;	•			CGG	;	ACG	CTC	:	CTC	:					TCG	CCG
			AGA					AGA			CTA		CTA						AGT	1
			AGO	3				AGG	;		CTG	+	CTG	;					AGC	;

									-	170										180
	CYS	ALA	TRP	GLU	VAL	VAL	ARG	ALA	GLU	ILE	MET	ARG	SER	PHE	SER	LEU	SER	THR	ASN	LEU
	TGY	GCN	TGG	GAR	GTN	GTN	MGN	GCN	GAR	АТН	ATG	MGN	WSN	TTY	WSN	YTN	WSN	ACN	AAY	YTN
5	TGT	GCT	TGG	GAA	GTT	GTT	CGT	GCT	GAA	ATT	ATG	CGT	TCT	TTT	TCT	TTA	TCT	ACT	AAT	TTA
	TGC	GCC		GAG	GTC	GTC	CGC	GCC	GAG	ATC		CGC	TCC	TTC	TCC	TTG	TCC	ACC	AAC	TTG
		GCA			GTA	GTA	CGA	GCA		ATA		CGA	TCA		TCA	CTT	TCA	ACA		CTT
		GCG			GTG	GTG	CGG	GCG				CGG	TCG		TCG	CTC	TCG	ACG		CTC
							AGA					AGA	AGT		AGT	CTA	AGT			CTA
10							AGG					AGG	AGC		AGC	CTG	AGC			CTG

| GLN | GLU | SER | LEU | ARG | SER | LYS | GLU | \*\*\*\*
CAR	GAR	WSN	YTN	MGN	WSN	AAR	GAR	TRR
15	---	---	---	---	---	---	---	
CAA	GAA	TCT	TTA	CGT	TCT	AAA	GAA	TAA
CAG	GAG	TCC	TTG	CGC	TCC	AAG	GAG	TAG
TCA	CTT	CGA	TCA	TGA				
TCG	CTC	CGG	TCG					
AGT	CTA	AGA	AGT					
AGC	CTG	AGG	AGC					

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Delivery and expression of nucleic acids in many formulations is limited due to degradation of the nucleic acids by components of organisms, such as nucleases. Thus, protection of the nucleic acids when delivered in vivo can greatly enhance the resulting expression, thereby enhancing a desired pharmacological or therapeutic effect. It was found that certain types of compounds which interact with a nucleic acid (e.g., DNA) in solution but do not condense the nucleic acid provide in vivo protection to the nucleic acid, and correspondingly enhance the expression of an encoded gene product.

We have described the use of delivery systems designed to interact with plasmids and protect plasmids from rapid extracellular nuclease degradation [Mumper, R.J., et al.,

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1996, Pharm. Res. 13:701-709; Mumper, R.J., et al., 1997. Submitted to Gene Therapy]. A characteristic of the PINC systems is that they are non-condensing systems that allow the plasmid to maintain flexibility and diffuse freely throughout the muscle while being protected from nuclease degradation. While the PINC systems are primarily discussed below, it will be understood that cationic lipid based systems and systems utilizing both PINCS and cationic lipids are also within the scope of the present invention.

A common structural component of the PINC systems is 10 amphiphilic molecules, shaving both that they are hydrophilic and a hydrophobic portion. The hydrophilic portion of the PINC is meant to interact with plasmids by hydrogen bonding (via hydrogen bond acceptor or Van der Waals interactions, or/and by ionic groups), 15 For example, PVP and N-methyl-2-pyrrolidone interactions. (NM2P) are hydrogen bond acceptors while PVA and PG are hydrogen bond donors.

All four molecules have been reported to form complexes with various (poly)anionic molecules [Buhler V., 20 39-42; Aktiengescellschaft Feinchemie, Ludwigshafen, Galaev Y, et al., J. Chrom. A. 684:45-54 (1994); Tarantino R, et al. J. Pharm. Sci. 83:1213-1216 (1994); Zia, H., et The hydrophobic Res. 8:502-504 (1991);]. Pharm. portion of the PINC systems is designed to result in a 25 plasmid rendering its surface the coating Kabanov et al. have described previously the hydrophobic. for plasmid cationic polyvinyl derivatives condensation designed to increase plasmid hydrophobicity, protect plasmid from nuclease degradation, and increase its 30 [Kabanov, for biological membranes A.V., affinity Kabanov, V.A., 1995, Bioconj. Chem. 6:7-20; Kabanov, A.V., et al., 1991, Biopolymers 31:1437-1443; Yaroslavov, A.A., et al., 1996, FEBS Letters 384:177-180].

35 Substantial protective effect is observed; up to at least a one log enhancement of gene expression in rat muscle

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over plasmid formulated in saline has been demonstrated with these exemplary PINC systems. We have also found that the expression of reporter genes in muscle using plasmids complexed with the PINC systems was more reproducible than when the plasmid was formulated in saline. 5 For example, the coefficient of variation for reporter gene expression in muscle using plasmid formulated in saline was  $96 \pm 35\%$  (n = 20 studies; 8-12 muscles/study) whereas with coefficient of variation with plasmids complexed with PINC systems was 40  $\pm$ 19% (n = 30 studies; 8-12 muscles/study). coefficient of variation for reporter gene expression with plasmid formulated in saline has been described previously [Davis, H.L., et al., 1993, Hum. Gene Ther. 4:151-9]. addition, in contrast with the results for DNA:saline, there was no significant difference in gene expression in muscle when plasmid with different topologies were complexed with polyvinyl pyrrolidone (PVP). This suggests that PVP is able to protect all forms of the plasmid from rapid nuclease degradation.

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## 20 1. Summary of interactions between a PINC polymer (PVP) and plasmid

We have demonstrated using molecular modeling that an exemplary PINC polymer, PVP, forms hydrogen bonds with the base pairs of a plasmid within its major groove and results in a hydrophobic surface on the plasmid due to the vinyl backbone of PVP. These interactions are supported by the modulation of plasmid zeta potential by PVP as well as by inhibition of ethidium bromide intercalation complexed plasmid. We have correlated apparent binding between PVP and plasmid to pH and salt concentration and have demonstrated the effect of these parameters on  $\square$ -gal expression after intramuscular injection of plasmid/PVP complexes [Mumper, R.J., et al., 1997. Submitted to Gene A summary of the physico-chemical properties of plasmid/PVP complexes is listed in Table I below.

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Table I: Summary of the Physico-Chemical Properties of Plasmid/PVP Complexes

Method	Result
Molecular modeling	Hydrogen bonding and
Fourier-transformed	hydrophobic plasmid surface
Infra-red Hydrogen	observed bonding demonstrated
DNase I challenge	Decreased rate of plasmid
	degradation in the presence
	of PVP
Microtitration	Positive heats of reaction
Calorimetry	indicative of an endothermic
	process
Potentiometric titration	One unit pH drop when plasmid
	and PVP are complexed
Dynamic Dialysis	Rate of diffusion of PVP
	reduced in the presence of
	plasmid
Zeta potential	Surface charge of plasmid
modulation	decreased by PVP
Ethidium bromide	Ethidium bromide
Intercalation	intercalation reduced by
	plasmid/PVP complexation
Osmotic pressure	Hyper-osmotic formulation
	(i.e., 340 mOsm/kg $H_2O$ )
Luminescence	Plasmid/PVP binding decreased
Spectroscopy	in salt and/or at pH 7

#### 2. Histology of expression in muscle

Immunohistochemistry for  $\beta\text{-gal}$  using a slide scanning technology has revealed the uniform distribution of  $\beta\text{-gal}$  expression sites across the whole cross-sections of rat tibialis muscles. Very localized areas were stained positive for  $\beta\text{-gal}$  when CMV- $\beta\text{-gal}$  plasmid was formulated in saline.  $\beta\text{-gal}$  positive cells were observed exclusively around the needle tract when plasmid was injected in saline. This is

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in agreement with previously published results [Wolff, J.A., et al., 1990, Science 247:1465-68; Davis, H.L., et al., 1993, Hum. Gene Ther. 4:151-9; Davis, H.L., et al., 1993, Hum. Gene Ther. 4:733-40].

In comparison, immunoreactivity for  $\beta$ -gal was observed 5 area of muscle tissue after intramuscular injection of CMV- $\beta$ -gal plasmid/PVP complex (1:17 w/w) in 150 It appeared that the majority of positive muscle mM NaCl. fibers were located at the edge of muscle bundles. 10 staining for  $\beta\text{-gal}$  in rat muscle demonstrated that, using a plasmid/PVP complex, the number of muscle fibers stained positive for  $\beta$ -gal was approximately 8-fold greater than found using a saline formulation. Positively stained muscle fibers were also observed over a much larger area in the muscle tissue using the plasmid/PVP complex providing 15 evidence that the injected plasmid was widely dispersed after intramuscular injection.

We conclude that the enhanced plasmid distribution and expression in rat skeletal muscle was a result of both 20 protection from extracellular nuclease degradation due to complexation and hyper-osmotic effects of the plasmid/PVP However, Dowty and Wolff et al. have demonstrated that osmolarity, up to twice physiologic osmolarity, did not significantly effect gene expression in muscle [Dowty, M.E., 25 Wolff, J.A. In: J.A. Wolff (Ed.), 1994, Gene Therapeutics: Methods and Applications of Direct Birkhauser, Boston, pp. 82-98]. This suggests that enhanced expression of plasmid due complexation is most likely due to nuclease protection and 30 less to osmotic effects. Further, the surface modification of plasmids by PVP (e.g., increased hydrophobicity decreased negative surface charge) may also facilitate the uptake of plasmids by muscle cells.

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### 3. Structure-activity relationship of PINC polymers

linear relationship between have found a structure of a series of co-polymers of vinyl pyrrolidone and vinyl acetate and the levels of gene expression in rat We have found that the substitution of some vinyl pyrrolidone monomers with vinyl acetate monomers in PVP resulted in a co-polymer with reduced ability to form hydrogen bonds with plasmids. The reduced interaction subsequently led to decreased levels of gene expression in rat muscle after intramuscular injection. The expression of  $\beta$ -gal decreased linearly (R = 0.97) as the extent of vinyl pyrrolidone monomer (VPM) content in the co-polymers decreased.

These data demonstrate that pH and viscosity are not the most important parameters effecting delivery of plasmid to muscle cells since these values were equivalent for all complexes. These data suggest that enhanced binding of the PINC polymers to plasmid results in increased protection and bioavailability of plasmid in muscle.

#### 4. Additional PINC systems

The structure-activity relationship described above can be used to design novel co-polymers that will also have enhanced interaction with plasmids. It is expected that there is "an interactive window of opportunity" whereby enhanced binding affinity of the PINC systems will result in gene expression after their further enhancement of intramuscular injection due to more extensive protection of plasmids from nuclease degradation. It is expected that there will be an optimal interaction beyond which either condensation of plasmids will occur or "triplex" result in decreased which can formation, either of muscle and consequently reduced gene bioavailability in expression.

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As indicated above, the PINC compounds are generally amphiphilic compounds having both a hydrophobic portion and hydrophilic portion. In many cases the hydrophilic portion is provided by a polar group. It is recognized in the art that such polar groups can be provided by groups such as, but not limited to, pyrrolidone, alcohol, acetate, amine or heterocyclic groups such as those shown on pp. 2-73 and 2-74 of CRC Handbook of Chemistry and Physics (72nd Edition), David R. Lide, editor, including pyrroles, pyrazoles, imidazoles, triazoles, dithiols, oxazoles, (iso)thiazoles, oxadiazoles, oxatriazoles, diaoxazoles, oxathioles, pyrones, dioxins, pyridines, pyridazines, pyrimidines, pyrazines, piperazines, (iso)oxazines, indoles, indazoles, carpazoles, and purines and derivatives of these groups, hereby incorporated by reference.

Compounds also contain hydrophobic groups which, in the case of a polymer, are typically contained in the backbone of the molecule, but which may also be part of a nonpolymeric molecule. Examples of such hydrophobic backbone groups include, but are not limited to, vinyls, acrylates, acrylamides, esters, celluloses, amides, hydrides, ethers, carbonates, phosphazenes, sulfones, propylenes, and derivatives of these groups. The polarity characteristics of various groups are quite well known to those skilled in the art as illustrated, for example, by discussions of polarity in any introductory chemistry textbook.

The ability of such molecules to interact with nucleic acids is also understood by those skilled in the art, and can be predicted by the use of computer programs which model such intermolecular interactions. Alternatively or addition to such modeling, effective compounds can readily identified using one or more of such tests as 1) determination of inhibition of the rate of nuclease digestion, 2) alteration of the zeta potential of the DNA, which indicates coating of DNA, 3) or inhibition of the

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ability of intercalating agents, such as ethidium bromide to intercalate with DNA.

#### 5. Targeting Ligands

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In addition to the nucleic acid/PINC complexes described above for delivery and expression of nucleic acid sequences, in particular embodiments it is also useful to provide a targeting ligand in order to preferentially obtain expression in particular tissues, cells, or cellular regions or compartments.

Such a targeted PINC complex includes a PINC system (monomeric or polymeric PINC compound) complexed to plasmid (or other nucleic acid molecule). The PINC system is covalently or non-covalently attached to (bound to) a targeting ligand (TL) which binds to receptors having an affinity for the ligand. Such receptors may be on the surface or within compartments of a cell. Such targeting provides enhanced uptake or intracellular trafficking of the nucleic acid.

The targeting ligand may include, but is not limited to, galactosyl residues, fucosal residues, mannosyl residues, carnitine derivatives, monoclonal antibodies, polyclonal antibodies, peptide ligands, and DNA-binding proteins. Examples of cells which may usefully be targeted include, but are not limited to, antigen-presenting cells, hepatocytes, myocytes, epithelial cells, endothelial cells, and cancer cells.

Formation of such a targeted complex is illustrated by the following example of covalently attached targeting ligand (TL) to PINC system:

TL-PINC + Plasmid -----> TL-PINC:::::Plasmid

Formation of such a targeted complex is also
illustrated by the following example of non-covalently
attached targeting ligand (TL) to PINC system
TL:::::PINC + Plasmid -----> TL:::::PINC:::::Plasmid
or alternatively,

PINC + Plasmid -----> PINC:::::Plasmid + TL ------> TL:::::PINC:::::Plasmid

In these examples :::::: is non-covalent interaction such as ionic, hydrogen-bonding, Van der Waals interaction,

- 5 hydrophobic interaction, or combinations of such interactions.
  - A targeting method for cytotoxic agents is described in Subramanian et al., International Application No. PCT/US96/08852, International Publication No. WO 96/39124,
- hereby incorporated by reference. This application describes the use of polymer affinity systems for targeting cytotoxic materials using a two-step targeting method involving zip polymers, i.e., pairs of interacting polymers.
- An antibody attached to one of the interacting polymers binds to a cellular target. That polymer then acts as a target for a second polymer attached to a cytotoxic agent. As referenced in Subramanian et al., other two-step (or multi-step) systems for delivery of toxic agents are also described.
- 20 In another aspect, nucleic acid coding sequences can be delivered and expressed using a two-step targeting approach involving a non-natural target for a PINC system or PINCtargeting ligand complex. Thus, for example, a PINC-plasmid complex can target a binding pair member which is itself attached to a ligand which binds to a cellular target (e.g., 25 Binding pairs for certain of MAB). the compounds identified herein as PINC compounds as identified Subramanian et al. Alternatively, the PINC can be complexed to a tareting ligand, such as an antibody. That antibody can be targeted to a non-natural target which binds to, for 30 example, a second antibody.

### III. Model Systems for Evaluation of Interferon Alpha Constructs and Formulations

In accord with the concept of using interferon alpha 35 expressing plasmid constructs and formulations in anti-

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cancer treatment, murine model systems were utilized based on murine tumor cell lines. The line primarily used was S.C. VII/SF, which is a cell line derived from murine squamous cell carcinoma (S.C.).

Squamous cell carcinoma of the head and neck begins with the cells lining the oral and pharyngeal cavities. Clinical disease progresses via infiltration and spreads lymphatics. underlying tissues and the undifferentiated, in vivo passage tumor line S.C. VII/SF displays this typical growth pattern. In addition, rapid growth rate provides a relatively short test period for individual experiments. Other murine tumor cell lines include another SCC line KLN-205, a keratinocyte line I-7, and a colon adenocarcinoma line MC-38.

optimal model system preferably satisfies criteria based on having tumor growth rate in vivo (i.e., tumors are ready for treatment in 4-10 days post implant), invasiveness, and local spread similar to those observed in providing accessibility and clinical disease, experimental treatment. As indicated, the SCC VII/SF cell 20 line was utilized as the primary model system cell line. This cell line typically grows rapidly, resulting in death of untreated syngeneic mice 14-17 days after tumor cell implantation.

This cell line can be utilized in a variety of ways to 25 provide model system suitable for a variety of different tests. Four such possibilities are described below.

First, SCCVII cells can be utilized in cell culture to vitro evaluation of interferon provide in an expression construct and formulation characteristics, such as expression levels and cellular toxicities.

Second, the cells can be implanted subcutaneously in This system can be utilized in tests in accessibility of the implant site is beneficial. example, the method was utilized evaluations in

expression efficiencies based on the expression of chloramphenical acetyltransferase (CAT).

Third, the cells can be implanted transcutaneously into the fascia of digastric muscle.

Fourth, the cells can be implanted transcutaneously into digrastric/mylohyoid muscles. The important features of models 3 and 4 are shown in the table below.

TABLE II: Comparison of submandibular tumor models

Feature	Mouse Tumor Model 3	Mouse Tumor Model 4					
Tumor implant	2-4 x 10 <sup>5</sup> cells	5 x 10 <sup>5</sup>					
procedure	transcutaneously into	transcutaneously into					
	fascia of digastric	digastric/mylohyoid					
	muscle	muscles					
Tumor growth and	Prominent	More variable,					
invasiveness	submandibular bulge;	invasion of					
characteristics	invasion of	digastric/mylohyoid					
	digastric/mylohyoid	muscles and lymphatics					
	muscles and						
<del></del>	lymphatics						
Treatment	Transcutaneous,	Lower jaw skin flap					
procedure	needle inserted and	raised to expose					
(primary	moved within tumor to	tumor, needle inserted					
treatment)	produce a 4 quadrant	and moved within tumor					
•	distribution of gene	to produce a 4					
	medicine	quadrant distribution					
		of gene medicine					
Days treated	Day 5, day 10 (both	Day 5 (tumor exposed),					
(post-implant)	transcutaneously)	day 8					
		(transcutaneously)					
Measurement	External calipering	First caliper when					
procedure	2-3 x per week until	tumor exposed for					
	death	treatment, second					
		caliper at sacrifice					

Feature	Mouse Tumor Model 3	Mouse Tumor Model 4
Advantages	Non-surgical, closed	Surgical, open model
	model allows larger	allows direct
	experiments and more	treatment of exposed
	frequent treatments;	tumor; Local
	Sacrifice unnecessary	inflammation from
	to caliper (=more	surgery may
	time points)	additionally stimulate
		immune response; More
	·	like clinical
<u>{</u> }		situation for protocol
		development
Disadvantages	Transcutaneous	Labor intensive;
	treatment is	Smaller, fewer
	potentially less	experiments possible;
:	accurate and	Tumors deeper and more
	intensive; less like	difficult to treat
	expected clinical	transcutaneously (for
	treatments than	secondary treatments);
	surgical approaches	Fewer treatments and
		caliperings possible

The tumor size treated in the mouse models is generally 20-50 mm<sup>3</sup>. A 50 mm<sup>3</sup> mouse tumor is approximately equivalent to 150 cc<sup>3</sup> human tumor having an average diameter of about 6.6 cm. This tumor size is approximately 10-fold larger than the size proposed to be treated in the phase I clinical trials. This indicates that the mouse models are strongly biased towards over estimating the expected tumor burden in human patients.

#### IV. Formulations for *In Vivo* Delivery

#### A. <u>General</u>

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While expression systems such as those described above provide the potential for expression when delivered to an appropriate location, it is beneficial to provide the expression system construct(s) in a delivery system which

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can assist both the delivery and the cellular uptake of the construct. Thus, this invention also provides particular formulations which include one or more expression system constructs (e.g., DNA plasmids as described above), and a protective, interactive non-condensing compound.

An additional significant factor relating to the plasmid construct is the percentage of plasmids which are in a supercoiled (SC) form rather than the open circular (OC) form.

#### B. Delivery and Expression

A variety of delivery methods can be used with the constructs and formulations described above, in particular, delivery by injection to the site of a tumor can be used. The submandibular tumor models utilized injection into four quadrants of the tumor being treated.

# C. Anti-Cancer Efficacy of Human Interferon Alpha Formulations

The effects of the administration of the interferon alpha formulations described above were evaluated using the S.C. VII mouse tumor models. Plasmid constructs as described above were incorporated in delivery formulations. The formulations were delivered by injection.

# D. Synergistic Effects of Interferon Alpha plasmid and IL-12 Plasmid and Effect of Human Interferon Alpha Formulation Administration on Production of Secondary Cytokines

The effects of the expression of the human interferon alpha plasmids in tumor cells on the progress of the mouse tumors demonstrates that such interferon alpha expression is effective against such tumors. However, it was also shown that IL-12 can act synergistically with the interferon alpha expression to exercise the antitumor effect (see Figure 9).

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### E. Toxicity Evaluation of Exemplary Formulations

The exemplary formulations do not show high cellular toxicity at the concentrations tested, suggesting that the formulations do not significantly kill cells by direct toxic action in vivo. Moreover, the anti-tumor activity induced by IFN $\alpha$  gene therapy is dependent upon activation of the immune system, which is demonstrated by depletion studies in vivo. Removal of a specific T lymphocyte population (CD8 $^+$ ) abrogates the anti-tumor activity elicited by IFN $\alpha$  gene therapy.

#### V. Administration

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Administration as used herein refers to the route of introduction of a plasmid or carrier of DNA into the body. In addition to the methods of delivery described above, the expression systems constructs and the delivery system formulations can be administered by a variety of different methods

Administration can be directly to a target tissue or by targeted delivery to the target tissue after systemic administration. In particular, the present invention can be used for treating disease by administration of the expression system or formulation to the body in order to establishing controlled expression of any specific nucleic acid sequence within tissues at certain levels that are useful for gene therapy.

The preferred means for administration of vector (plasmid) and use of formulations for delivery are described above. The preferred embodiments are by direct injection using needle injection.

The route of administration of any selected vector construct will depend on the particular use for the expression vectors. In general, a specific formulation for each vector construct used will focus on vector uptake with regard to the particular targeted tissue, followed by demonstration of efficacy. Uptake studies will include

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uptake assays to evaluate cellular uptake of the vectors and expression of the DNA of choice. Such assays will also determine the localization of the target DNA after uptake, and establishing the requirements for maintenance of steady-state concentrations of expressed protein. Efficacy and cytotoxicity can then be tested. Toxicity will not only include cell viability but also cell function.

Muscle cells have the unique ability to take up DNA from the extracellular space after simple injection of DNA particles as a solution, suspension, or colloid into the muscle. Expression of DNA by this method can be sustained for several months.

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Delivery of formulated DNA vectors involves incorporating DNA into macromolecular complexes that undergo endocytosis by the target cell. Such complexes may include lipids, proteins, carbohydrates, synthetic compounds, or inorganic compounds. Preferably, the complex includes DNA, a cationic lipid, and a neutral lipid in particular proportions. The characteristics of the complex formed with the vector (size, charge, surface characteristics, composition) determines the bioavailability of the vector within the body. Other elements of the formulation function as ligand which interact with specific receptors on the surface or interior of the cell. Other elements of the formulation function to enhance entry into the cell, release from the endosome, and entry into the nucleus.

Delivery can also be through use of DNA transporters. DNA transporters refers to molecules which bind to vectors and are capable of being taken up by epidermal DNA transporters contain a molecular complex capable of noncovalently binding to DNA and efficiently transporting the DNA through the cell membrane. It is preferable that the transporter also transport the DNA through the nuclear See, e.g., the following applications all of membrane. which (including drawings) hereby are incorporated by reference herein: (1) Woo et al., U.S. Serial

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07/855,389, entitled "A DNA Transporter System and Method of Use,, filed March 20, 1992, now abandoned; (2) Woo et al., PCT/US93/02725, International Publ. WO93/18759, entitled "A DNA Transporter System and Method of Use", (designating the and other countries) filed March 19, 5 continuation-in-part application by Woo et al., entitled "Nucleic Acid Transporter Systems and Methods of Use", filed December 14, 1993, U.S. Serial No. 08/167,641; (4) Szoka et al. , U.S. Serial No. 07/913,669, entitled "Self-Assembling Polynucleotide Delivery System", filed July 14, 1992 and (5) 10 Szoka et al., PCT/US93/03406, International Publ. WO93/19768 entitled "Self-Assembling Polynucleotide Delivery System", (designating the U.S. and other countries) filed April 5, A DNA transporter system can consist of particles containing several elements that are independently and non-15 covalently bound to DNA. Each element consists of a ligand which recognizes specific receptors or other functional groups such as a protein complexed with a cationic group Examples of cations which may be used that binds to DNA. derivatives, histone, spermine, spermine 20 peptides and/or polylysine. One element is capable binding both to the DNA vector and to a cell surface receptor on the target cell. Examples of such elements are organic compounds which interact with the asialoglycoprotein folate receptor, the mannose-6-phosphate receptor, the 25 receptor, or the carnitine receptor. A second element is capable of binding both to the DNA vector and to a receptor on the nuclear membrane. The nuclear ligand is capable of recognizing and transporting a transporter system through a nuclear membrane. An example of such ligand is the nuclear 30 targeting sequence from SV40 large T antigen or histone. third element is capable of binding to both the DNA vector and to elements which induce episomal lysis. include inactivated virus particles such as adenovirus, peptides related to influenza virus hemagglutinin, or the 35 GALA peptide described in the Szoka patent cited above.

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Transfer of genes directly into a tumor has been very effective. Experiments show that administration by direct injection of DNA into tumor cells results in expression of the gene in the area of injection. Injection of plasmids containing human interferon alpha results in expression of the gene for 5 days following a single intra-tumoral injection. Human IFN $\alpha$  production was highest in tumors harvested 1 day post-tumor injection and steadily declined thereafter. The injected DNA appears to persist in an unintegrated extrachromosomal state. This means of transfer is a preferred embodiment.

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Administration may also involve lipids as described in preferred embodiments above. The lipids may form liposomes which are hollow spherical vesicles composed of lipids arranged unilamellar, bilamellar, in or multilamellar fashion and an internal aqueous space for entrapping water soluble compounds, such as DNA, ranging in size from 0.05 to several microns in diameter. Lipids may be useful without forming liposomes. Specific examples include the use of cationic lipids and complexes containing DOPE which interact with DNA and with the membrane of the target cell to facilitate entry of DNA into the cell.

Gene delivery can also be performed by transplanting genetically engineered cells. For example, immature muscle cells called myoblasts may be used to carry genes into the muscle fibers. Myoblast genetically engineered to express recombinant human growth hormone can secrete the growth hormone into the animal's blood. Secretion of the incorporated gene can be sustained over periods up to 3 months.

Myoblasts eventually differentiate and fuse to existing muscle tissue. Because the cell is incorporated into an existing structure, it is not just tolerated but nurtured. Myoblasts can easily be obtained by taking muscle tissue from an individual who needs gene therapy and the genetically engineered cells can also be easily put back with out causing damage to the patient's muscle. Similarly,

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keratinocytes may be used to delivery genes to tissues. numbers of keratinocytes can generated be cultivation of a small biopsy. The cultures can be prepared as stratified sheets and when grafted to humans, generate epidermis which continues to improve in histotypic quality The keratinocytes are many years. by transfecting culture in engineered while the appropriate vector. Although keratinocytes with keratinocytes are separated from the circulation by the basement membrane dividing the epidermis from the dermis, human keratinocytes secrete into circulation the protein produced.

of delivery should result chosen method expression of the gene product encoded within the nucleic acid cassette at levels which an appropriate exert biological effect. The rate of expression will depend upon the disease, the pharmacokinetics of the vector and gene product, and the route of administration, but should be in range 0.001-100 mg/kg of body weight /day, preferably 0.01-10 mg/kg of body weight/day. This level is readily determinable by standard methods. It could be more or less depending on the optimal dosing. The duration of treatment will extend through the course of the disease symptoms, possibly continuously. The number of doses will depend upon the disease, delivery vehicle, and efficacy data from clinical trials.

#### Examples

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The present invention will be more fully described in conjunction with the following specific examples which are not to be construed in any way as limiting the scope of the invention. As shown below, mIFN- gene medicine reduces the growth of tumors in syngeneic murine tumor models. Lipid formulations of mIFN- gene medicine display anti tumor activity in both SCC-VII and MC-38 tumor models. PINC and peptide formulations of mIFN- gene medicine display anti

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tumor effects in the MC-38 tumor model. The anti tumor effects of mIFN- gene medicine are dose dependent. In addition, the examples demonstrate that treatment of tumors with the combination of IFN $\alpha$  and IL-12 gives an unanticipated more than additive (synergystic) anti-tumor activity using either a PINC or a lipid formulation.

#### Example 1

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A plasmid expression system encoding murine IFNlpha4 and formulated in a polymeric delivery system was used for in vivo immunotherapeutic activity against an 10 immunogenic murine renal cell carcinoma, Renca, and a non-immunogenic mammary adenocarcinoma, TS/A. Mice bearing established tumors were treated with  $IFN\alpha/polyvinyl-pyrrolidone$  (PVP) expression complexes via direct intra-tumoral injection. to 100 % tumor growth inhibition was observed in the treated 15 By using an optimal dose of 96 and 48  $\mu g$ formulated IFN- $\alpha$  plasmid for the treatment of Renca and TS/A respectively, 30% (Renca) and 10% (TS/A) of the treated animals remained tumor-free. Tumor inhibition was dependent upon activation of the immune system. The anti-tumor 20 activity elicited by IFN- $\alpha$  gene therapy was abrogated when mice were selectively depleted of CD8 T cells. By contrast, removal of CD4<sup>+</sup> resulted in increased tumor rejection following IFN- $\alpha$ /PVP treatments. Finally, mice that remained tumor-free following IFN- $\alpha$  gene therapy displayed immune 25 resistance to a subsequent challenge of tumor. These data provide evidence that non-viral IFN $\alpha$  gene therapy can be used to induce an efficient anti-tumor response.

Local presence of cytokines in tumors can activate an immune response that in some cases leads to induction of specific long-lasting anti-tumor immunity. By direct intratumoral injection of plasmid encoding murine IFNα4 and formulated in a polymeric delivery system, tumor-bearing mice develop an immune response, which leads to inhibition and eradication of the tumor. We have shown by depletion

studies in vivo that the immune response induced by IFN $\alpha$  is mainly CD8-mediated and that this treatment results in a long-term immunity in mice demonstrating complete tumor regression. Thus, non-viral IFN $\alpha$  gene therapy may be an effective alternative to IFN $\alpha$  protein therapy for human cancers.

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Transduction of tumor cells with cytokine genes has proven to be a very efficient technique to induce cytokinemediated anti-tumor immunity. In experimental models, the local presence of IL-2, IL-1, IL-4, IL-6, IL-7, IL-12, IFNs 10 and CSFs (i.e., GM-CSF) at the site of the tumor can result in significant tumor growth inhibition (Colombo et al., "Local Cytokine Availability Elicits Tumor Rejection and Systemic Immunity Through Granulocyte-T-Lymphocyte Cross-Talk", Cancer Research, 52, 4853-4857 (1992)). In these 15 limited effect on have cytokines systems, proliferation directly but are capable of activating a rapid and potent anti-tumor immune response, which impedes tumor Established parental tumors, however, progression. difficult to eradicate with ex vivo cytokine-transduced 20 tumor cells because efficacy of vaccination is dependent on the size, growth rate and invasiveness of the tumor.

To overcome these problems, cytokine-based gene therapy approaches, which can deliver transgenic cytokines locally 25 and induce an anti-tumor immune response, have been recently evaluated by a number of investigators (Forni et al., "Cytokine-Induced Immunogenicity: From Exogenous Cytokines to Gene Therapy", Journal of Immunotherapy, 14, 253-257, "An Efficient Th2-type Memory Pericle et al., (1993);30 Cd8+ Lymphocyte-driven and Eosinophil-mediated Rejection of a Spontaneous Mouse Mamary Adenocarcicoma Engineered to Release I1-4", The Journal of Immunology, 153, 5660-5673. (1994); Pardoll et al., "Gene Modified Tumor Vaccines, In Cytokine-Induced Tumor Immunogenicity", eds. 35 Academic Press, London, p. 71-86. (1994); and Musiani et

al., "Cytokines, Tumor-cell Death and Immunogenicity: A Question of Choice", Immunology Today. 1, 32-36 (1997)). Technological breakthroughs in gene therapy adenoviral, retroviral, and liposomal vectors have provided powerful tools with which to study the biological effects of specific cytokine mediators as well as to develop novel and clinically applicable anti-tumor immunotherapies (Pardoll, "Paracrine Cytokine Adjuvants in Cancer Immunotherapy", Annu. Rev. Immunol. 13, 399-415 (1995); Bramson et al., "Direct Intratumoral Injection of an Adenovirus Expressing 10 Interleukin-12 Induces Regression and Long-lasting Immunity That Is Associated with Highly Localized Expression of Interleukin-12", Hum. Gene Ther., 7, 1995-2002 (1996); Rao et al., "Il-12 Is an Effective Adjuvant to Recombinant Vaccinia Virus-based Tumor Vaccines", J. Immunol. 15 3357-3365. 1996; Rakhmilevich et al., "Gene Gun-mediated Transfection with Interleukin 12 Gene Results Regression of Established Primary and Metastatic Murine Tumors", Proc. Natl. Acad. Sci. USA. 93, 6291-6296 (1996); 20 and Rakhmilevich et al, "Cytokine Gene Therapy of Cancer Using Gene Gun Technology: Superior Antitumor Activity of Interleukin-12", Hum. Gene Ther. 8, 1303-1311, (1997)).

gene therapy approach utilizing an interactive polymeric gene delivery system that increases expression by protecting plasmid DNA (pDNA) from nucleases and controlling the dispersion and retention of pDNA in muscle cells is described in Mumper et al., 1996. polymeric interactive non-condensing (PINC) systems routinely result in a greater amount of gene expression from tissues as compared to delivery of unformulated plasmid in saline (Mumper et al., 1996). By using a plasmid that encodes insulin human growth factor-1 (hIGF-1) formulated as a PINC complex, production of biologically active h IGF-1 in vivo following intra-muscular injection has been shown (Alila et al., "Expression of Biologically Active Human Insulin-Like Growth Factor-1 Following

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Intramuscular Injection of a Formlated Plasmid in Rats", Human Gene Therapy, 8, 1785-1795 (1997)). The specific objective of this study was to determine whether a plasmid expression system encoding murine IFNlpha4 and formulated as a complex with PVP could induce an anti-tumor immune response following direct injection into subcutaneous murine tumors.

The IFN family consists of three major glycoproteins, IFN $\alpha$ , IFN $\beta$  and IFN $\gamma$ . Although IFNs were first developed as antiviral agents, it is now clear that they also control growth and differentiation, and modulate various 10 aspects of host immunity (Gresser et al., "Antitumor effects of interferon", Acta Oncol. 28, 347-353 (1989)). data concluded that systemic chronic administration of  $\mathsf{IFN}\alpha$ could produce regression of vascular tumors, hemangiomastosis, Kaposi's pulmonary sarcoma, hemangiomas (Singh et al., "Interferons A and B Downregulate the Expression of Basic Fibroblast Growth Factor in Human Carcicomas", Proc. Natl. Acad. Sci. USA. 92, 4562-4566 (1995)). Although IFN $\alpha$  was the first cytokine to be used in clinical trials that proved to be effective against certain types of human cancer, only recently has this cytokine been considered as a candidate for gene therapy (Ogura et al. Belldegrun et al., "Human Renal Carcinoma Line Transfected With Interleukin-2 and/or Interferon  $\alpha$  Gene(s): Implications for Live Cancer Vaccines, <u>Journal of the</u> National Cancer Institute, 85, 207-216 (1993).

Initial studies have shown that the injection of genetically modified tumor cells producing  $IFN\alpha$ syngeneic mice induces tumor growth inhibition and elicits a tumor-specific immune memory (Ferrantini et al., Interferon Alpha-1-Interferon Gene Transfer into Metastatic Friend Lukemia Cells Abrogated Tumorigenicity in Immunocompetent Mice: Antitumor Therapy by Means of Interferon-Producing Cells; Cancer Res. 53, 1107-4615 (1993); Ferrantini et al., a Metastatic Expression into Gene Adenocarcicoma (Ts/a) Results in Cd8+ T Cell-Mediated Tumor

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Rejection and Development of Antitumor Immunity: Comparative Studies with Ifn- $\gamma$ -producing Ts/a Cells" Journal of Immunology, 153, 4604-4615, (1994); Musiani et al. 1997). However, the real value of this potential form of vaccine in inducing the regression of established tumors remains to be demonstrated.

In this study we present evidence that direct injection of IFN $\alpha$  plasmid formulated in PVP into subcutaneous murine tumors results in a host-dependent tumor rejection, primarily mediated by CD8 $^+$  T cells, and elicits a protective immunity against subsequent tumor re-challenge.

#### Materials And Methods

### Plasmid construction and formulation

plasmid system containing an expression expression cassette for mIFN-1 $\alpha4$  was constructed as follows. 15 The coding sequence of the murine IFN- $\alpha4$  gene (Genebank X01973 M15456 M23830 X01967) was amplified by PCR from mouse genomic DNA. amplified mIFN- $\alpha4$  sequence was then subcloned into a plasmid backbone, and the sequence fidelity was verified by DNA sequence 20 analysis (data not shown). The coding sequence for mIFN- $\alpha4$  was then subcloned as an XbaI-BamHlfragment into the expression plasmid pIL0697 to create the mIFN- $\alpha4$  expression system pIF0836. Plasmid pVC0612 (empty plasmid, EP) contains expression elements including the cytomegalovirus immediate early promoter and the 3' UTR/poly(A) signal from the bovine growth gene in the pVC0289 25 backbone described by Alila et al. (1997). Plasmid pVC0612 was used as a control plasmid in all in vivo experiments. for intra-tumoral injection were grown under kanamycin selection in E. coli host strains DH5lpha and purified using conventional 30 alkaline lysis and chromatographic methods. Purified plasmid utilized for intra-tumoral injections had the following specifications: endotoxin (< 500 Eu/mg plasmid); protein (< 1%); and chromosomal DNA (< 20 %). Purified pIF0836 and control

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plasmids were formulated at a concentration of 3 mg DNA/ml in a solution of 5 % w/v polyvinyl-pyrrolidone (Plasdone C-30, ISP Technologies, Wayne, NJ), 150 mM NaCl on the day of injection, as described previously (Mumper et al., 1996).

### 5 Western blot analysis and bioassay for mIFN $\alpha$ .

HeLa cells were plated in 6 well plates at 3  $\times$   $10^5$  cells per well, and transfected using 1  $\alpha\mu g$  of mouse IFN $\alpha 4$  plasmid pIF0836C and 3  $\mu g$  of Lipofectamine (Life Technologies, Inc., Same supernatants Gaithersburg, MD) in serum-free DMEM. were harvested 24 hours later and immunoprecipitated using anti-mouse interferon  $\alpha/\beta$  polyclonal antibody (BioSource International, Camarillo, CA) and protein A and G agarose (Boehringer Mannheim, Indianapolis, IN). Samples were run on a 12% Tris-glycine gel and electroblotted to Millipore PVDF membrane. Anti-mouse interferon  $\alpha/\beta$  polyclonal antibody 1:1000, followed by anti-sheep at used 1:1000. Biotinylated molecular (Boehringer Mannheim) at Streptavidin-HRP detected using were markers weight (Amersham, Arlington Heights, IL). Detection was performed using the Amersham ECL kit. Supernatants were also tested for IFN $\alpha$  biological activity using L929 cells treated with encephalomyocarditis virus, in parallel with a NIH mouse IFN $\alpha$  reference reagent (Access Biomedical, San Diego, CA).

#### Animals

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Normal 8-week-old female BALB/c mice were purchased from Harlan Laboratories, Houston, TX. Mice were maintained on ad libitum rodent feed and water at 23°C, 40% humidity, and a 12-h/12-h light-dark cycle. Animals were acclimated for at least 4 days before the start of the study.

#### 30 Tumors

Three established mouse tumor models were used in this study. TS/A is a tumor cell line established by Dr. P. Nanni, University of Bologna, Italy, from the first in vivo

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transplant of a moderately differentiated adenocarcinoma that spontaneously arose in a BALB/c mouse A number of pre-immunizational., 1983). challenge experiments suggested that TS/A does not elicit a long-lasting anti-tumor immunity (Forni et al., 1987). was generously provided by Dr. Guido Forni, University of Turin, Italy. Renca, a spontaneously arising murine renal cell carcinoma, and CT-26, a colon adenocarcinoma, generously provided by Dr. Drew M. Pardoll, John Hopkins 10 Hospital, Baltimore, MD. Tumor cell cultures maintained in sterile disposable flasks from (Corning, NY) at  $37^{\circ}$  C in a humidified 5%  $CO_2$  atmosphere, using RPMI 1640 supplemented with 10% FBS. 100 penicillin, 100 U/ml streptomycin and 50  $\mu$ g/ml gentamycin; 15 all from Life Technologies.

### In vivo evaluation of tumor growth and treatments

BALB/c mice were challenged s.c. in the middle of the left flank with 30  $\mu l$  of a single-cell suspension contained the specified number of cells. Seven days later when the tumor size reached approximately  $10 \text{ mm}^3$ , treatments with 20 IFN $\alpha$ /PVP or EP/PVP started and were repeated at 1-2 day intervals for 2 weeks (total of 8 treatments: 4/week). Tumor volume was measured with electronic caliper in the two perpendicular diameters and in the depth. Measurements of the tumor masses  $(mm^3)$  were performed twice a week for 40-5025 All mice bearing tumor masses exceeding 1 cm3 volume were sacrificed for humane reasons. When depletion of immunocompetent cells in vivo was required, a group of mice received i.v 0.5 ml of  $\alpha\text{-CD4}$  (GK1.5 hybridoma, 207-TIB, 30 Rockville, MD) ascite (1:10), or α-CD8 hybridoma, 210-TIB, ATCC) ascite (1:100) or i.p. 100  $\mu g$ (RB6-8C5 hybridoma, Pharmingen, San Diego, Control mice received i.v. 0.5 ml isotype control (Pharmingen). Antibody treatments were performed twice: first injection 1 day before starting the gene therapy 35

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treatment and the second injection (i.p at the same dosage) 7 days later.

#### CTL assay

A standard 6-hour 51-chromium (51Cr)-release assay was performed following 5 days of in vitro effector cell 5 Single cell suspensions of splenocytes were stimulation. prepared 3 weeks following tumor challenge by mashing the spleens in RPMI 1640 medium (Life Technologies) and passing the cells through 70 µm nylon mesh cell strainers (Falcon, Becton Dickinson, Lincoln Park, NJ) into 50ml centrifuge 10 tubes (Falcon). After centrifugation, red blood cells were lysed with ACK Lysing Buffer (Biofluids, Inc., Rockville, MD) and the splenocytes washed twice with RPMI. In vitro stimulation cultures contained 3 X 106 splenocytes/effectors per ml with 6 X 10<sup>5</sup> mitomycin-C-treated Renca/stimulator 15 cells per ml and 10 Units per ml recombinant murine IL-2 (Genzyme, Cambridge, MA) in RPMI containing 10% FBS, 22mM HEPES buffer (Research Organics Inc., Cleveland OH), Penn-10<sup>-5</sup> M  $2-\beta$ -mercapto-ethanol Streptomycin, 5 Х Technologies), OPI media supplement (Sigma, St. Louis, MO), 20 amino acids and non-essential essential Technologies) (for a 5 : 1 responder : stimulator ratio). Stimulators were prepared by incubating Renca cells at 3 X  $10^7$  per ml in RPMI with 30  $\mu g$  per ml mitomycin-C (Sigma) at  $37^{0}$  C for 60 minutes, followed by four washes in HBSS with 25 After 5 days at  $37^{\circ}$  C, effector cells were 2.5% FBS. pelleted, resuspended in complete RPMI, counted, and mixed with  $^{51}Cr$  -labeled targets in a 96 well V-bottomed plate (Costar/Corning, Cambridge, MA). Renca and CT26 targets were labeled by incubating them at 2  $\times$   $10^6$  cells per ml in 30 complete RPMI with 150 uCi 51Cr (Amersham) for 2.5 hours. Targets were washed 3 times in HBSS with 2.5% FBS and resuspended in complete RPMI before addition to the assay. After mixing effectors and targets (in triplicate wells) and a brief pelleting, plates were placed at  $37^{\circ}$  C for 6 hours. 35

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Approximately 90% of the supernatants were then collected from each well with the Skatron Harvesting Press and Supernatant Collection System (Skatron Instruments, Norway). <sup>51</sup>Cr release was detected using a WALLAC 1470 Wizard automatic gamma counter (WALLAC Inc., Gaithersburg MD). Specific release was determined with the following equation: (experimental cpm - spontaneous cpm) / (total cpm-spontaneous cpm) X 100. Spontaneous release from the targets was less than 18%, and the standard error of the triplicate experimental counts was less than 14%.

#### Statistical analysis

Data for the effects of mIFN- $\alpha$  gene therapy on tumor growth were analyzed by repeated measures analysis. Individual treatment means were compared using Duncan's multiple range test when the main effect was significant. Data for the effect of mIFN- $\alpha$  gene therapy on tumor rejection were analyzed by ANOVA. In all cases a p value of less than 0.05 was considered to be statistically significant.

#### 20 Results

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#### Expression of mIFN- $\alpha$

IFN-α expression plasmid (pIF0836) transfected into Cos-1 cells, and the resulting conditioned assayed for mIFN- $\alpha$  by Western blot and by media was bioassay. Western blot analysis of conditioned media indicated that the recombinant mIFN- $\alpha$  expressed from pIF0836 template was present as a single band with an approximate molecular weight of 23 kDa. This band was not observed in conditioned media from mock-transfected cells and likely represents a glycosylated form of mIFN- $\alpha$ . Recombinant mIFN- $\alpha$  ran with an approximate molecular weight of 18 kDa, which corresponds to the predicted molecular weight of nonglycosylated mIFN- $\alpha$ . Assay of conditioned media using an

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anti-viral bioassay for mIFN- $\alpha$  indicated that approximately 175 x 10<sup>3</sup> IU/ml mIFN- $\alpha$  were present.

Anti-tumor activity of IFN- $\alpha$  gene therapy. The antitumor effect of murine IFNα4 plasmid formulated as a complex with PVP (IFN $\alpha$ /PVP) was tested in a syngeneic murine renal cell carcinoma (Renca) and a mammary adenocarcinoma (TS/A) BALB/c mice were challenged subcutaneously tumor model. with 7  $\rm X10^5$  Renca or 1  $\rm X10^5$  CT26 cells, and IFN $\alpha/PVP$ injections were initiated 7 days later when tumors reached approximately 10 mm<sup>3</sup> size. Each group of mice received at interval of 1-2 days 8 treatments (4 injections/week) of IFN $\alpha$ /PVP at scalar doses (from 12 to 96  $\mu$ g/mouse), EP/PVP (96  $\mu$ g/mouse) or no treatments for control (ctrl). size increased progressively in mice injected with EP/PVP (Renca, TS/A) or low doses of IFN $\alpha$ /PVP (TS/A), while tumors in mice injected with each dose of IFN $\alpha/PVP$  (Renca) or high dose of IFN $\alpha$ /PVP (TS/A) showed marked growth inhibition.

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# Tumor growth inhibition is associated to systemic immune response

Treatments of Renca and TS/A tumors with IFN $\alpha$ /PVP at 96 µg/mouse and 48 µg/mouse respectively, induced complete regression in 6 out of 20 (Renca) and 2 out of 20 (TS/A) of challenged mice. To test whether the rejection of these tumors leads to specific immune memory, mice with no tumors for 40-50 days following IFN $\alpha$  treatments were re-challenged with a greater number of fresh tumors in the right flank. All mice that rejected primary tumors displayed protection against the second tumor challenge whereas mice used as the control group and injected for the first time with same number of tumor cells (1 X 10<sup>6</sup> Renca or 2 X 10<sup>5</sup> TS/A) developed tumors.

To evaluate the requirements for the induction of antitumor immune memory, Renca and TS/A were injected into BALB/c rendered immunosuppressed by treatment with anti-CD4, anti-CD8 or anti-polymorphonuclear cells (PMN). Depletion

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of CD8<sup>+</sup> T cells allowed both Renca and TS/A to grow in all animals following IFN $\alpha$ /PVP treatments, showing that this population is crucial for the immune response induced by IFN $\alpha$  gene therapy. No increase in tumor growth was found in mice treated with anti-PMN ( $\alpha$ -GR1) monoclonal Ab (mAb). Increase in tumor rejection was observed in mice depleted of CD4<sup>+</sup> T and treated with IFN $\alpha$ /PVP suggesting that depletion of CD4<sup>+</sup> T cells can enhance the anti-tumor effect of IFN $\alpha$  gene therapy.

Expression of IFN- $\alpha$  within the tumor induces a CTL 10 To assess whether CD8+ tumor specific CTL were induced in vivo by IFN $\alpha$ /PVP treatments, splenocytes from Renca tumor-challenged mice were tested for their cytolytic activity following IFNa gene therapy. Cytotoxic activity against Renca, and not against CT26 cells used as control 15 for tumor specificity, was found in 2 of 4 mice that had received IFN $\alpha$  gene therapy. Moreover, splenocytes from mice of CD4<sup>+</sup> T cells and treated with demonstrated potent CTL activity against Renca cells. 20 contrast, little CTL activity against Renca cells evident from splenocytes isolated from mice treated with EP/PVP.

#### Discussion

The data reported herein demonstrate that administration of IFNα gene formulated 25 in a delivery system into subcutaneous renal cell carcinoma and mammary adenocarcinoma inhibits tumor growth and induces a long-lasting immunity to secondary tumor challenges. considerable significance is the fact that the anti-tumor response was observed against both an immunogenic carcinoma 30 well а more aggressive and poorly immunogenic adenocarcinoma, phenotype which is a similar to spontaneously arising tumors in man.

A variety of genetic abnormalities arise in human 35 cancers that contribute to neoplastic transformation and

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malignancy. Despite increasing understanding of the remain malignancies cancer, many molecular basis of resistant to established forms of treatment. More recently, molecular genetic interventions have been designed in an attempt to improve the efficacy of immunotherapy. numerous experimental studies have been performed in murine models with tumor cells transduced with cytokine-gene ex vivo, a major limitation in the translation of this strategy to large-scale human tumor vaccine therapy is the labor intensity and variability of establishing each individual tumor in culture and transducing it with an appropriate vector (i.e., retrovirus). Our approach to this problem is to use a non-viral delivery system to modify tumor cells in vivo with cytokine cDNAs so that the tumor cells can supply the cytokine of interest in a paracrine fashion to the antitumor responder cells present within the tumor.

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Using a plasmid containing IFN $\alpha4$  gene and formulated in PVP, we have shown that intra-tumoral injections of this DNA-PINC complex can lead to complete tumor regression in 30 % of the cases (Renca model) with an overall response rate 20 of 100 % tumor growth inhibition. These results are in agreement with a recent study that described an anti-tumor activity elicited by genetically modified TS/A producing murine IFN- $\alpha$ 1 (Ferrantini et al., 1994). Although the anti-tumor effect of IFN $\alpha$  using cytokine-gene transduced 25 described (Scarpa cells has been Matrix Remodelling in Murine Mamarv "Extracellular Adenocarcicoma Transfected with the Interferon-alphal Gene", Journal of Pathology. 181, 116-123 1997), the real value of IFNa gene therapy in blocking or inhibiting advanced tumors 30 remains to be explored. The advantage of using a non-viral IFNα gene delivery system over retrovirus is that tumor cells could be transduced directly in vivo without the need of first establishing tumor cells in vitro. Moreover, IFNa has a potent anti-viral activity limiting the use of this 35 gene in combination with viral vectors.

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Therapeutic levels of gene expression for IGF-I using a similar interactive PVP-based delivery system have previusly been described (Alila et al., 1997). Direct intra-tumor injection of the same PINC delivery system as a complex with IFN $\alpha$  gene, resulted in dispersion in vivo of plasmid into target cells inducing an IFN $\alpha$ -specific anti-tumor activity. Tumors treated with the same plasmid but in the absence of IFN $\alpha$  coding region and formulated as a complex with PVP, did not respond to this treatment and grew in a similar rate to untreated tumors. By using an optimal dose of  $IFN\alpha/PVP$ , tumor-bearing mice were able to reject the tumors mounting a specific long-lasting tumor immunity. Although, the numbers of mice rejecting a second tumor challenge was low, this observation indicates that a considerable portion of the activity of IFN $\alpha$  in inducing the rejection of established tumors is not anti-angiogenic or anti-proliferative but immunostimulatory. Our result demonstrating that induced regression of advanced tumors was prevented by in vivo administration of anti-CD8 mAb provides direct evidence for a key role of CD8 T cells in the anti-tumor effect of IFNα.

Depletion of CD4 T cells in tumor-bearing mice treated with IFNα gene therapy significantly enhanced therapeutic effect of IFNa, resulting in tumor regression and prolonged survival of up to 80% of treated mice. A CD4mediated suppression during tumor progression has previously reported and it has also been shown depletion of CD4 T cells in tumor-bearing mice results in augmentation of anti-tumor therapy with either IL-2 or IL-12 (Rackmilevich et al., 1994 and Martinotti et al., "Cd4 T Cells Inhibit in Vivo the Cd8-Mediated Immune Response Against Murine Colon Carcinoma Cells Transducted Interleukin-12 Genes", Eur. J. Immunol, 25, 137-146. They have shown that depletion of CD4 \*T cells in (1995)). tumor-bearing mice in the absence of treatment did not affect the growth of tumor itself suggesting that removal of

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CD4 T cells does not deprive the tumor of growth factors (Rackmilevich et al., 1994). A possible explanation for this phenomenon is that depletion of CD4 T cells from tumorbearing mice augments the anti-tumor efficacy of 5 CD8<sup>+</sup>  $\mathbf{T}$ activated cells by releasing them from immunosuppression. The mechanism driving CD4 suppression is poorly understood, although Th2 type cytokines, directly or cell activation, may inhibit cell-mediated immunity (Mossman et al., 1989; Powrie et al., 10 Immunol, 23(11):3043-9 (1993)). CTL can be generated in both CD4-depleted and non-depleted mice from lymphocytes from spleens by in vitro re-stimulation with mitomycin-treated Renca cells and rIL-2. Thus, CD4-mediated suppression appears to be exerted on CD8 expansion and not In accord with the in vivo results, stronger CTL 15 activity was observed from mice depleted of CD4 and treated with IFN $\alpha$ /PVP suggesting CD4 $^{+}$  T cells inhibit an IFN $\alpha$ mediated CD8 T cell response in vivo. This study suggests that direct administration of cytokine genes, like IFNa, 20 into tumors, which have been found to suppress malignancy growth, provide a new therapeutic option for the treatment of human cancers.

# Example 2: Pharmacology of mIFN - Gene Medicine in Syngeneic Tumor Models

Gene expression systems encoding either mIFN-2 or mIFN-4 were tested for anti tumor activity when formulated in either cationic lipid, peptide, or PINC delivery systems and injected intratumorally into subcutaneous squamous cell carcinoma (SCC-VII) or adenocarcinoma (MC-38) tumors.

#### 30 Experimental design and treatment regimen

Experiments to test the anti tumor activity of mIFN-gene medicine were conducted in either SCC-VII or MC-38 tumor models. Tumor cells  $(4 \times 10^5)$  were injected subcutaneously into the flank region of mice, and treatment

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was initiated when tumor volume reached approximately 50  $\text{mm}^3$ . Treatment was begun approximately 6 days (SCC-VII tumors) and 10 days (MC-38 tumors) after tumor initiation and repeated at 3 to 5 day intervals.

5 All formulations of mIFN- gene medicine were administered in a dose volume of 50 μl. The faster growing SCC-VII tumors typically received 3 treatments, whereas the relatively slower growing MC-38 tumors typically received 4 treatments. Experiments were terminated when lactose vehicle control tumors reached approximately 1000 mm<sup>3</sup>.

The anti-tumor effects of murine IFN gene medicine (IFNa/PVP) was tested in syngeneic murine renal cell carcinoma (Renca) and mammary adenocarcinoma (TS/A) tumor BALB/c mice were challenged subcutaneously with 7  $\rm X10^{5}$  or 1  $\rm X10^{5}$  CT26, and IFN $\alpha/\rm PVP$  injections were initiated 7 15 days later when tumors reached approximately 10 mm3 size. Each group of mice received 8 treatments (4 injections for 2 weeks) of IFN $\alpha$ /PVP at scalar doses (from 12 to 96  $\mu$ g/mouse), empty plasmid/PVP (EP/PVP, 96 µg/mouse) or no treatments for 20 control (ctrl). Tumor size increased progressively in mice injected with EP/PVP (Renca, TS/A) or low doses of  $IFN\alpha/PVP$ (TS/A), while tumors in mice injected with each dose of IFN $\alpha$ /PVP (Renca) or high dose of IFN $\alpha$ /PVP (TS/A) marked growth inhibition.

# Example 3: mIFN- Gene Medicine Formulated in Cationic lipid Reduces the Growth of SCC-VII Tumors

Experiments were conducted in the SCC-VII tumor model as described in the preceding example. mIFN- gene medicine formulated in cationic lipid, peptide, and PINC delivery systems was tested. Results show that cationic lipid formulations significantly reduce the growth of SCC-VII tumors relative both to lactose vehicle injected tumors and to tumors injected with control (non coding) plasmid formulated in cationic lipid. The effect of mIFN- gene medicine formulated in cationic lipid is dose dependent and

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there is no effect of mIFN- gene medicine when formulated in PVA. In addition, analysis of tumors from this experiment using immunohistochemical methods revealed infiltration of CD8+ lymphocytes in tumors injected with cationic lipid formulations, but not in tumors injected with PVA formulations.

mIFN- gene medicine significantly reduces the growth of SCC-VII tumors as compared to control plasmid or lactose injected tumors. Differences between control plasmid and mIFN- plasmid were consistent across formulation. Plasmid dose was 46  $\mu$ g/treatment. Growth of tumors injected with control plasmid was compared to that of tumors injected with mIFN- gene medicine using repeated measures analysis. mIFN- reduced SCC-VII tumor growth relative to control plasmid (p=.035).

# Example 4: mIFN- Gene Medicine Reduces the Growth of MC-38 Tumors

Experiments were carried out as described in the preceding examples. The effects of various prototype formulations of mIFN- gene medicine on the growth of subcutaneous MC-38 tumors were compared. mIFN- gene medicine elicited reduced tumor growth in all formulations tested (cationic lipid, peptide, and PINC). Subsequent experiments in the MC-38 tumor model have shown similar results.

#### Example 5: Dose Responses

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After demonstrating anti tumor effects of mIFN- gene medicine, the dose response for these effects was investigated in the MC-38 tumor model. Both cationic lipid (DOTMA:Chol) and PINC (PVA) delivery systems were evaluated. Results clearly show that mIFN- gene medicine elicited a dose dependent reduction in tumor growth. Tumor volume in this experiment was maximally reduced by approximately 50 % with mIFN- /DOTMA:Chol and 60 % with mIFN- /PVA after 4

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treatments. Maximal reduction in tumor volume was observed at a plasmid dose of approximately 50  $\mu g$ /treatment (cumulative dose of approximately 200  $\mu g$ ). These experiments will be conducted primarily in the MC-38 tumor model because it provides a broader treatment window than does the SCC-VII model.

#### Example 6: IFN-alpha Formulations

The formulations for the IFN-a are: (1) PVP 4 vial, (2) PVP three vial, (3) PVP two vial. The details are listed below:

#### PVP 4 vial

Materials: 25% PVP (50 kDa) stock solution, plasmid stock solution, 5 M NaCl stock solution, and water.

Method: Add in order of water, plasmid, 25% PVP and 5 M NaCl into a vial to make a plasmid in 5% PVP in saline formulation. The final concentration of PVP and NaCl are fixed (5% and 150 mM) and plasmid concentration could be varied (but based on the IGF-1 data, 3 mg DNA/ml in 5% PVP in saline should be the best formulation). The quality of the formulation is characterized by pH, DNA concentration, osmolality, and gel electrophoresis. The DNA concentration could be varied from 0.1-5 mg/ml. The pH may be varied from 3-5, osmolality may be 250 - 400 mOsm.

#### Three vial

Material: lyophilized PVP, plasmid stock solution (4 mg/ml), 115 mM Na-Citrate/5% NaCl stock buffer (pH = 4).

Method: Add in order of plasmid and buffer into the PVP to make final 3 mg DNA/ml in 5% PVP in 25 mM citrate/saline buffer (pH =4). DNA expression is higher in saline than in the citrate buffer.

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#### Two vial

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Materials: Co-lyophilized plasmid and PVP, saline. Add saline into the co-lyophilized DNA and PVP to make final 3 mg/ml DNA in 5% PVP in saline.

The final formulation is 3mg/mL DNA, 5% PVP as a single vial. The formulation is prepared by adding (5%) PVP to (4mg/mL) DNA and recirculating the two components for a finite period of time (using static mixer). Then the formulation is lyophilized and rehydrated with 0.9% sodium chloride, to obtain a final composition of 3mg/mL, 5%PVP in saline.

#### Example 7: Treatment of Human Tumors

The murine studies are predictive of the response of Human tumors to therapy using a plasmid construct encoding the human IFN alpha gene sequence such as that depicted in SEQ ID NO: 10, 11 or 12. A patient in need of anti-cancer therapy is injected with up to 3mg of plasmid formulation at daily intervals. The plasmid formulation may contain INF alpha plasmid alone or optionally a mixture of IFN-alpha encoding plasmid and an additional plasmid coding for a cytokine. The preffered cytokine is IL-12. The treatments are continued and the patient monitored as is the usual practice for anti-cancer chemotherapeutic regimes.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.

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It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from scope and spirit of the invention.

5 All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated reference.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the of such terms expressions of excluding and equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. For example,

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described as selected from the group consisting of bromine, chlorine, and iodine, claims for X being bromine and claims for X being bromine and chlorine are fully described.

Other embodiments are within the following claims.

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#### Claims

- 1. A plasmid comprising a CMV promoter transcriptionally linked with an interferon alpha coding sequence, and a growth hormone 3'-untranslated region.
- 5 2. The plasmid of claim 1, wherein said interferon alpha is human interferon alpha.
  - 3. The plasmid of claim 2, wherein said human interferon alpha coding sequence is a synthetic sequence having optimal codon usage.
- 4. The plasmid of claim 3, wherein said interferon alpha coding sequence has the nucleotide sequence of SEQ ID NO:10, 11 or 12.
- 5. The plasmid of claim 1, wherein said growth hormone 3' untranslated region is from a human growth hormone gene.
  - 6. The plasmid of claim 5, wherein an ALU repeat or ALU repeat-like sequence is deleted from said 3' untranslated region.
- 7. The plasmid of claim 1, wherein said plasmid 20 includes a promoter, a TATA box, a Cap site and a first intron and intron/exon boundary in appropriate relationship for expression of said coding sequence.
- 8. The plasmid of claim 7, wherein said plasmid further comprises a 5' mRNA leader sequence inserted between said promoter and said coding sequence.

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- 9. The plasmid of claim 1, wherein said plasmid further comprises an intron/5' UTR from a chicken skeletal  $\alpha$ -actin gene.
- 10. The plasmid of claim 1, wherein said plasmid 5 comprises a nucleotide sequence which is the same as the nucleotide sequence of plasmid pIF0921.
  - 11. The plasmid of claim 1, further comprising:
  - a first transcription unit comprising a first transcriptional control sequence transcriptionally linked with a first 5'-untranslated region, a first intron, a first coding sequence, and a first 3'-untranslated region/poly(A) signal, wherein said first intron is between said control sequence and said first coding sequence; and

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- a second transcription unit comprising a second transcriptional control sequence transcriptionally linked with a second 5'-untranslated region, a second intron, a second coding sequence, and a second 3'-untranslated region/poly(A) signal, wherein said second intron is between said control sequence and said second coding sequence;
- wherein said first and second coding sequences comprise a sequence having the sequence of SEQ ID NO:2, 3, 4 or 25 coding for human IL-12 p40 subunit, and a sequence having the sequence of SEQ ID NO:6, 7, 8 or 24 coding for human IL-12 p35 subunit.
- 25 The plasmid of claim 11, wherein said first transcriptional control sequence or said second transcriptional control sequence comprise one or more cytomegalovirus promoter sequences.
- 13. The plasmid of claim 11, wherein said first and 30 second transcriptional control sequences are the same.

- 14. The plasmid of claim 11, wherein said first and second transcriptional control sequences are different.
- 15. The plasmid of claim 14, wherein said sequence coding for the p40 subunit of human IL-12 is 5' to said 5 sequence coding for the p35 subunit of human IL-12.
  - 16. The plasmid of claim 1, further comprising an intron having variable splicing, a first coding sequence, and a second coding sequence,
- wherein said first and second coding sequences comprise a sequence having the sequence of SEQ ID NO:2, 3, 4 or 25 coding for human IL-12 p40 subunit, and a sequence having the sequence of SEQ ID NO:6, 7, 8 or 24 coding for human IL-12 p35 subunit.
  - 17. The plasmid of claim 16, further comprising:
- a transcriptional control sequence transcriptionally linked with a first coding sequence and a second coding sequence;
  - a 5'-untranslated region;
  - an intron 5' to said first coding sequence;
- an alternative splice site 3' to said first coding sequence and 5' to said second coding sequence; and
  - a 3'-untranslated region/poly(A) signal.
- 18. The plasmid of claim 17, wherein said transcriptional control sequence comprises a cytomegalovirus 25 promoter sequence.
  - 19. The plasmid of claim 1, further comprising:
- a transcriptional control sequence transcriptionally linked with a first coding sequence, an IRES sequence, a second coding sequence, and a 3'-30 untranslated region/poly(A) signal, wherein said IRES

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sequence is between said first coding sequence and said second coding sequence; and

an intron between said promoter and said first coding sequence;

- wherein said first and second coding sequences comprise a sequence having the sequence of SEQ ID NO:2, 3, 4 or 25 coding for human IL-12 p40 subunit, and a sequence having the sequence of SEQ ID NO:6, 7, 8 or 24 coding for human IL-12 p35 subunit.
- 10 20. The plasmid of claim 19, wherein said transcriptional control sequence comprises a cytomegalovirus promoter sequence.
  - 21. The plasmid of claim 19, wherein said IRES sequence is from an encephalomyocarditis virus.
- 22. A composition comprising the plasmid of anyone of claims 1-21, and a protective, interactive non-condensing compound.
- 23. The composition of claim 22, wherein said protective, interactive non-condensing compound is polyvinyl 20 pyrrolidone.
  - 24. The composition of claim 22, wherein said plasmid is in a solution having between 0.5% and 50% PVP.
  - 25. The composition of claim 24, wherein said solution includes about 5% PVP.
- 25 26. The composition of claim 22, wherein said DNA is at least about 80% supercoiled.
  - 27. The composition of claim 26, wherein said DNA is at least about 90% supercoiled.

- 28. The composition of claim 27, wherein said DNA is at least about 95% supercoiled.
- 29. A composition comprising a protective, interactive non-condensing compound and a plasmid comprising an interferon alpha coding sequence.
  - 30. A composition comprising the plasmid of any one of claims 1-21 and a cationic lipid with a neutral colipid.
- 31. The composition of claim 30, wherein said cationic 10 lipid is DOTMA.
  - 32. The composition of claim 30, wherein said neutral co-lipid is cholesterol.
- 33. The composition of claim 30, wherein the DNA in said plasmid and said cationic lipid are present in such amounts that the negative to positive charge ratio is about 1:3.
  - 34. The composition of claim 30, wherein said DNA is at least about 80% supercoiled.
- 35. The composition of claim 34, wherein said DNA is 20 at least about 90% supercoiled.
  - 36. The composition of claim 35, wherein said DNA is at least about 95% supercoiled.
  - 37. The composition of claim 30, further comprising an isotonic carbohydrate solution.

- 38. The composition of claim 37, wherein said isotonic carbohydrate solution consists essentially of about 10% lactose.
- 39. The composition of claim 30 wherein said cationic 5 lipid and said neutral co-lipid are prepared as a liposome having an extrusion size of about 800 nanometers.

### 40. A composition comprising:

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- a first component comprising a plasmid comprising an interferon alpha coding sequence and a cationic lipid with a neutral co-lipid, wherein said cationic lipid is DOTMA and said neutral co-lipid is cholesterol, wherein the DNA in said plasmid and said cationic lipid are present in amounts such that the negative to positive charge ratio is about 1:3; and
- a second component comprising a protective, interactive non-condensing compound, wherein said first component is present within the second component.
  - 41. A composition comprising a protective, interactive non-condensing compound, a first plasmid comprising an interferon alpha coding sequence, and one or more other plasmids independently comprising an IL-12 or IL-12 subunit coding sequence.
- 42. A composition comprising a plasmid comprising an interferon alpha coding sequence and a cationic lipid with a neutral co-lipid.
  - 43. A method for making a plasmid of anyone of claims 1-21 comprising the step of inserting a CMV promoter transcriptionally linked with an interferon alpha coding sequence, and a growth hormone 3'-untranslated region into a plasmid.

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- 44. A method for making a composition of claim 29, comprising the steps of:
- a. preparing a DNA molecule comprising a transcriptional unit, wherein said transcriptional unit comprises an interferon alpha coding sequence;
- b. preparing a protective, interactive non-condensing compound; and
- c. combining said protective, interactive noncondensing compound with said DNA in conditions such that a 10 composition capable of delivering a therapeutically effective amount of an interferon alpha coding sequence to a mammal is formed.
- 45. The method of claim 44 wherein said DNA molecule is a plasmid, wherein said plasmid comprises a CMV promoter transcriptionally linked with an interferon alpha coding sequence, and a human growth hormone 3'-untranslated region/poly(A) signal.
  - 46. A method of making a composition of claim 30, comprising the steps of:
- 20 a. preparing a DNA comprising an interferon alpha coding sequence;
  - b. preparing a mixture of a cationic lipid and a neutral co-lipid, wherein said cationic lipid is DOTMA and said neutral co-lipid is cholesterol; and
- c. combining said mixture with said DNA in amounts such that said cationic lipid and said DNA are present in a negative to positive charge ratio of about 1:3.
  - 47. A method of making a composition of claim 40, comprising the steps of:
- a. preparing a first component comprising a plasmid comprising an interferon alpha coding sequence and a cationic lipid with a neutral co-lipid, wherein said cationic lipid is DOTMA and said neutral co-lipid is

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cholesterol, wherein the DNA in said plasmid and said cationic lipid are present in amounts such that the negative to positive charge ratio is about 1:3;

- b. preparing a second component comprising a protective, interactive non-condensing compound; and
  - c. combining said first and second components such that the resulting composition comprises said first component within said second component.
- 48. A method of making a composition of claim 41, 10 comprising the steps of:
  - a. preparing a protective, interactive noncondensing compound,
  - b. preparing a first plasmid comprising an interferon alpha coding sequence,
- 15 c. preparing one or more other plasmids independently comprising an IL-12 p35 or IL-12 p40 subunit coding sequence, and
- d. combining said protective, interactive noncondensing compound, said plasmid comprising said interferon
   alpha coding sequence and said other plasmids.
  - 49. A method of making a composition of claim 42 comprising combing a plasmid comprising a interferonalpha coding sequence and a cationic lipid with a neutral colipid.
- 50. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a plasmid of anyone of claims 1-21.
- 51. The method of claim 50, wherein said condition or 30 disease is a cancer.

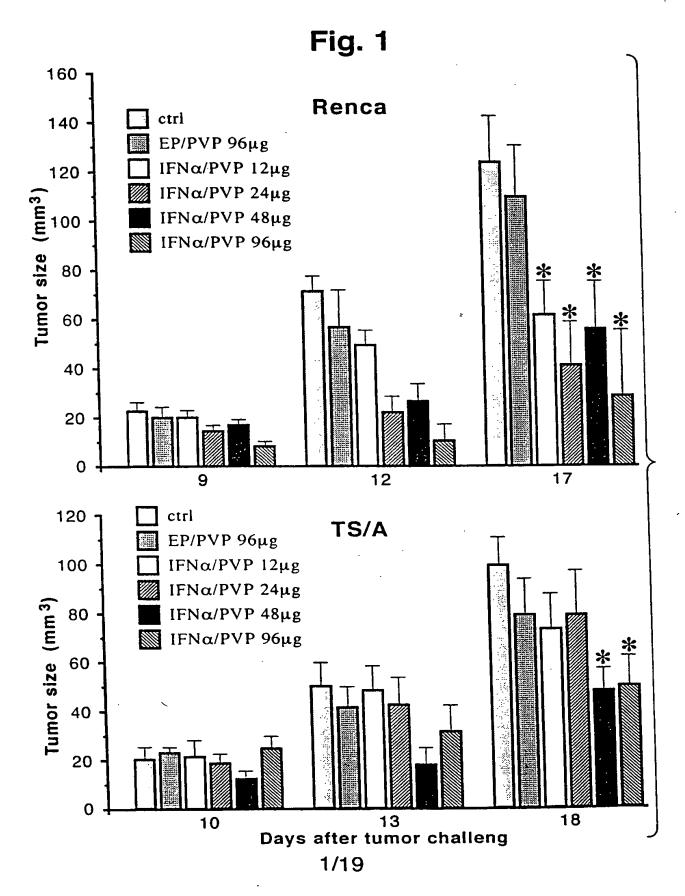
- 52. The method of claim 50, wherein said composition is administered by injection.
- 53. A method for transfection of a cell in situ, comprising the step of contacting said cell with a plasmid of anyone of claims 1-21 for sufficient time to transfect said cell.
  - 54. The method of claim 53, wherein transfection of said cell is performed in vivo.
- 55. The method of claim 53, wherein said contacting is performed in the presence of an about 5% PVP solution.
  - 56. A method for delivery and expression of an interferon alpha gene in a plurality of cells, comprising the steps of:
- (a) transfecting said plurality of cells with a plasmid of anyone of claims 1-21; and
  - (b) incubating said plurality of cells under conditions allowing expression of a nucleic acid sequence in said vector, wherein said nucleic acid sequence encodes interferon alpha.
- 57. The method of claim 56, wherein said interferon alpha is human interferon alpha and said cells are human cells.
  - 58. The method of claim 56, wherein said contacting is performed in the presence of an about 5% PVP solution.
- 59. A method for treating a disease or condition, comprising the steps of transfecting a cell *in situ* with a plasmid of any one of claims 1-21.

- 60. The method of claim 59, wherein said disease or condition is a localized disease or condition.
- 61. The method of claim 59, wherein said disease of condition is a systemic disease or condition.
- 5 62. A cell transfected with a plasmid of anyone of claims 1-21.
- 63. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of claim 22.
  - 64. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of claim 29.
- 15 65. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of claim 30.
- 66. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of claim 40.
- 67. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of claim 41.
  - 68. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from

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said condition or disease a therapeutically effective amount of a composition of claim 42.

69. A method for treatment of a mammalian condition or disease, comprising administering to a mammal suffering from said condition or disease a therapeutically effective amount of a composition of a first plasmid comprising an interferon alpha coding sequence and a second plasmid comprising a IL-12 coding sequence.



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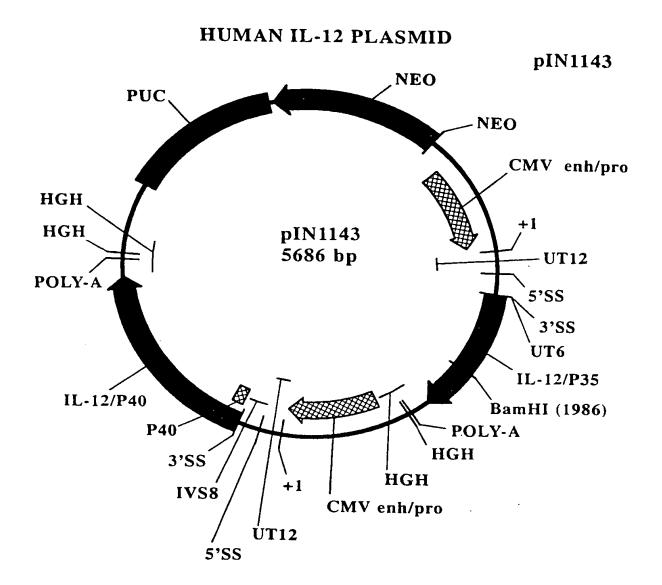


Fig. 2A

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CAGTGACTATTGATAGAGTGATGAGCTATCTGAATGCTTCCTAACAATTCTAGAAAAGCCGAATTCTGCAGGAATTGGGTGGCATCCCTGTGACCCCTCCCAG AATTTCAACAGTGAGACTGTGCCACAAAAATCCTCCCTTGAAGAACCGGATTTTTTATAAAACTAAAATCAAGCTCTGCATACTTCTTCATGCTTTCAGAATTCGGG GAGACCTCTTTCATAACTAATGGGAGTTGCCTGGCCTCCAGAAAGACCTCTTTTATGATGGCCCTGTGCCTTAGTAGTATTTATGAAGACTTGAAGATGTACCAGG GAAGAGATTGATCATGAAGATATCACAAAAGATAAAACCAGCACAGTGGAGGCCTGTTTACCATTGGAATTAACCAAGAATGAGAGTTGCCTAAATTCCAGA AGGAATGTTCCCATGCCTTCACCACTCCCAAAACCTGCTGAGGGCCGTCAGCAACATGCTCCAGAAGGCCAGACAAACTCTAGAATTTTACCCCTTGCACTTCT GCCACCATGGGTCCAGCGCGCAGCCTCCTCCTTGTGGCTACCCTGGTCCTCCTGGACCACCTCAGTTTGGCCAGAAACCTCCCCGTGGCCACTCCAGACCC

TGGAGTTCAAGACCATGAATGCAAAGCTTCTGATGGATCCTAAGAGGCAGATCTTTCTAGATCAAAACATGCTGGCAGTTATTGATGAGCTGATGCAGGCCCTG

*ACAGGTAAGTGTCTTCCTCCTGTTTCCTTCCCCTGCTATTCTGCTCAACCTTCCTATCAGAAACTGCAGTATCTGTATTTTTTGCTAGAATTGTACTAACGGTTCTTTTTTT* CGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCGATCCAGCCTCCGCGGCGGGGAACGGTGCATTGGAACGCGGATTCCCCGTGTTAATT*I* GTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGA CATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTTGGCAGTACATCAATGI ITATGTCGTAGAATTGGATTGGTATCCGGATGCCCCTGGAGAAATGGTGGTCCTCACCTGTGACACCCCTGAAGAAGATGGTATCACCTGGACCTTGGACCAG GGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGO GGTGGAGGGGGGTGGTATGGAGCAAGGGGGCAAGTTGGGAAGACAACCTGTAGGGCTCGAGGGGGGGCCCGGTACGGTCGTTACATAACTTACGGTAA*t* TGGCCCGCCTGGCTGACCGCCCAACGACCCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATC TGCCTCTCCTGGCCCTGGAAGTTGCCACTCCAGTGCCCACCAGCCTTGTCCTAATAAAATTAAGTTGCATCATTTTGTCTGACTAGGTGTCCTTCTATAATATTATGI 4/19

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AATTAAGTTGCATCATTTTGTCTGACTAGGTGTCCTTCTATAATATTATGGGGTGGAGGGGGGGTGGTATGGAGCAAGGGGCAAGTTGGGAAGACAACCTGTA(

GGCTCGAGGGGGGCCCGGTACCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTTCGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATC(

GCAAAAATGCCAGCATTAGCGTGCGGGCCCAGGACCGCTACTATAGCTCATCTTGGAGCGAATGGGCATCTGTGCCCTGCAGTTAGACGCGCTAGAAAA

**AAACCTGACCCACCCAAGAACTTGCAGCTGAAGCCATTAAAGAATTCTCGGCAGGTGGAGGTCAGCTGGGAGTACCCTGACACCTGGAGTACTCCACATT** 

GGGTGACGTGCGGAGCTGCTACACTCTCTGCAGAGAGAGTCAGAGGGGGACAACAAGGAGTATGAGTACTCAGTGGAGTGCCAGGAGGACAGTGCCTGI CCAGCTGCTGAGGAGAGTCTGCCCATTGAGGTCATGGTGGATGCCGTTCACAAGCTCAAGTATGAAAACTACACCAGCAGCTTCTTCATCAGGGACATCAT

GCCAAGAATTATTCTGGACGTTTCACCTGCTGGTGGCTGACGACAATCAGTACTGATTTGACATTCAGTGTCAAAAGCAGCAGCAGAGGCTCTTCTGACCCCCAAG A GCA GT GA GGT CTTA GGCT CT GGCAAAA CCCT GA CCAT CCAA GT CAAA GA GTTT GGA GA T GCT GGCCA GTA CA CCT GT CA CAA

CCGAATTCTGCAGGAATTGGGTGGCATCCCTGTGACCCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTCCAGTGCCCACCAGCCTTGTCCTAAT*ai* 

GTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGAAGAACTCGTCAAGAAGGCGC **ACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGG** CAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCC CCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTC AAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGAC AAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTTGCTGGCGTTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCAC TAGAAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTCTTCAGCAATATCACGGGTA GCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAG

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Codon Frequency

human\_high.doc 143

### Codon usag for human (highly xpressed) genes 1/24/91

AmAcid	Codon	Number	/1000	Fraction
Gly	GGG	905.00	18.76	0.24
Gly	GGT	441.00	9.14	0.12
Gly	$\mathbf{G}\mathbf{G}\mathbf{C}$	1867.00	38.70	0.50
Glu	GAG	2420.00	50.16	0.75
Glu	GAA	792.00	16.42	0.25
Asp	GAT	592.00	12.27	0.25
Asp	GAC	1821.00	37.75	0.75
Val	GTG	1866.00	38.68	0.64
Val	GTA	134.00	2.78	0.05
Val	GTT	198.00	4.10	0.01
Val	GTC	728.00	15.09	0.25
Ala	GCG	652.00	13.51	0.17
Ala	GCA	488.00	10.12	0.13
Ala	GCT	654.00	13.56	0.17
Ala	GCC	2057.00	42.64	0.53
Arg	AGG	512.00	10.61	0.18
Arg	$\mathbf{A} \mathbf{G} \mathbf{A}$	298.00	6.18	0.10
Ser	AGT	354.00	7.34	0.10
Ser	AGC	1171.00	24.27	0.34
Lys	AAG	2117.00	43.88	0.82
Lys	$\mathbf{A} \mathbf{A} \mathbf{A}$	471.00	9.76	0.18
Asn	AAT	314.00	6.51	0.22
Asn	AAC	1120.00	23.22	0.78
Met	ATG	1077.00	22.32	1.00
Ile	ATA	88.00	1.82	0.05
Ile	ATT	315.00	6.53	0.18
Ile	ATC	1369.00	28.38	0.17
Thr	ACG	405.00	8.40	0.15
Thr	ACA	373.00	7.73	0.14
Thr	ACT	358.00	7.42	0.14
Thr	ACC	1502.00	31.13	0.57
Тгр	TGG	652.00	13.51	1.00
End	TGA	109.00	2.26	0.55
Cys	TGT	325.00	6.74	0.32
Cys	TGC	706.00	14.63	0.68
End	TAG	42.00	0.87	0.21
End	TAA	46.00	0.95	0.23
Tyr	TAT	360.00	7.46	0.26
Туг	TAC	1042.00	21.60	0.74

Fig. 3

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### MOUSE INTERFERON ALPHA

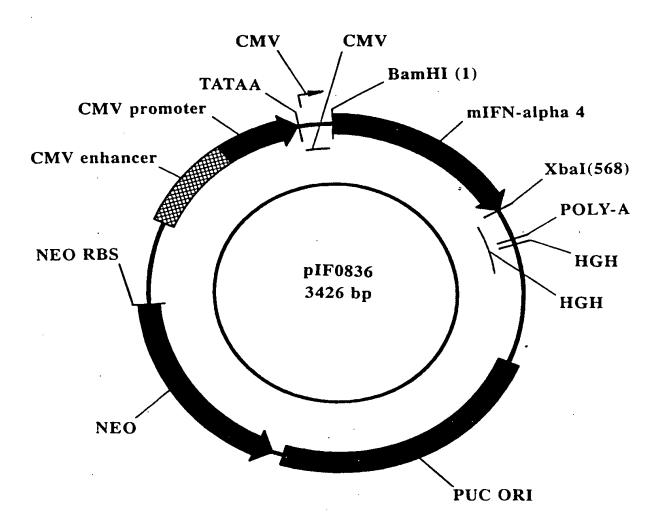


Fig. 4A

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GCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCCTGATGCTCTTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCATCCGAGTACGTGCTC

AGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGG AAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAA

GTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAA GCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTAT GTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGC

CCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGGCATCGCCATGCGTCACGACGAGATCCTCGCCGTCGGGGCATGC

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**AAAAAGGCCGCGTTGCTGGCGTTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGAC** 

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GAGCAAGGTGAGATGACAGGAGATCCTGCCCCGGCACTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAG GCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGATCAAGCGTATGCAGCCGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAG ATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTT GACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTT AAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACAT CCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGT CTGCGTGCAATCCATCTTGTTCAATCATGCGAAACGATCCTCATCCTGTCTCTTGATCAGATCTTGATCCCCTGCGCCATCAGATCCTTGGCGGCAAGAAAGCCA CCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCAGCCGATTGTCTGTTGTGCCCAGTCATAGCCGAATAGCCTCTCCACCCAAGCGGCCGGAGAAC TCCAGTTTACTTTGCAGGGCTTCCCAACCTTACCAGAGGGCGAATTCGAGCTTGCATGCCTGCAGGTCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGA 

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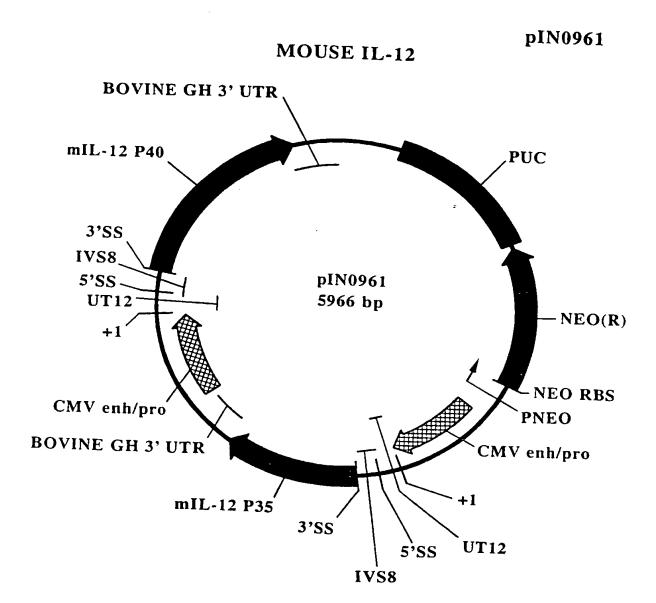


Fig. 5A

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# ig. 5C

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ig. 5D

# pIF0921 **HUMAN IFN**

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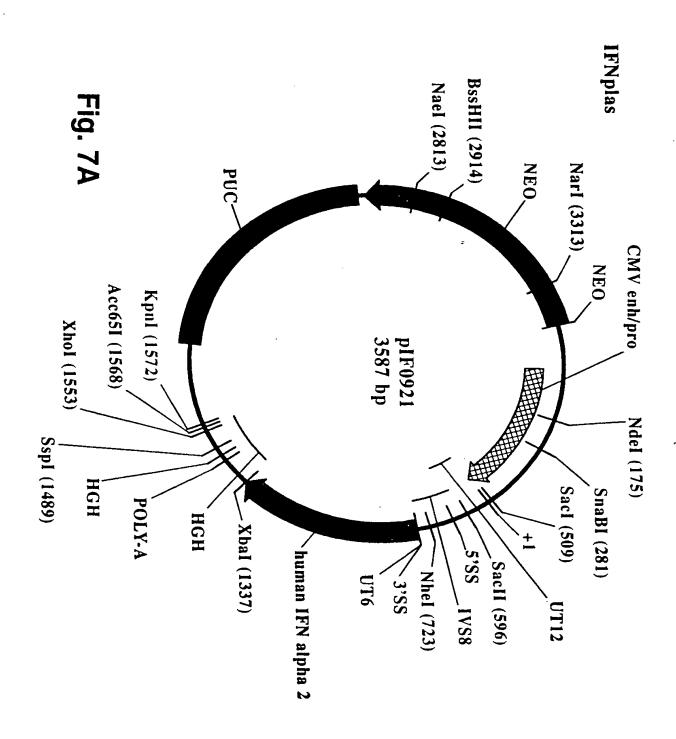
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SUBSTITUTE SHEET (RULE 26)

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ig. 6B

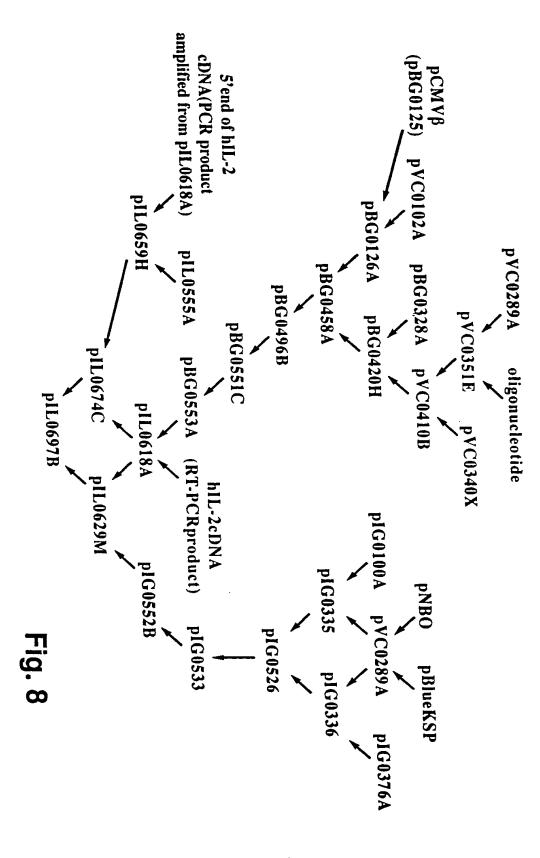
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16/19
SUBSTITUTE SHEET (RULE 26)

# DNA coding sequence for IFN-02b gene in pIF0921

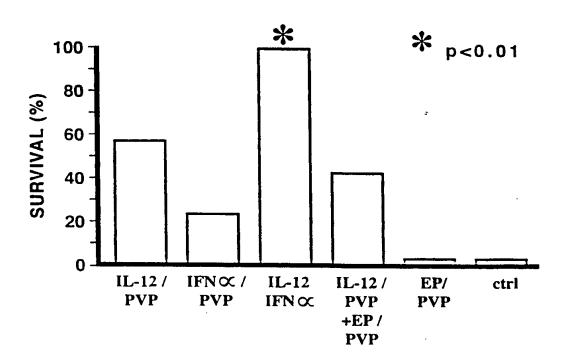
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18/19

## IL-12 Gene Medicine (Combination Therapy) in Renca Model

IL-12 / IFN-∝



IL-12 / PVP (24  $\mu$ g) IL-2 / DC (6  $\mu$ g) IFN-  $\propto$  (96  $\mu$ g) EP= empty plasmid / PVP (96  $\mu$ g)

Fig. 9

1

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PCT/US99/05394

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	ACCRORDED A	<b>™™~~~~™~~~~~</b>	<u> </u>	GCTTTCCCAGT	CGGGWWWCCI	GICGIGCCEC	240
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		· ^m^***	י רכאידראידנא:	A GACATCACAU	. GGGACCAAA	CUGCUCUITO	3120 3180
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_	GCGTGTACGG TGGGAGGTCT ATATAAGCAG AGCTCGTTTA GTGAACCGTC AGATCGCCTG	4440
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	GCTGTGAGG	A AATACTICC	C DCCDCDDDT	C ATGAGATOT	T TTTCTTTGT	C AACAAACTTG	540
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        WSNCARAAYY TNYTNMGNGC NGTNWSNAAY ATGYTNCARA ARGCNMGNCA RACNYTNGAR TTYTAYCCNT GYACNWSNGA RGARATHGAY CAYGARGAYA THACNAARGA YAARACNWSN ACNGTNGARG CNTGYYTNCC NYTNGARYTN ACNAARAAYG ARWSNTGYYT NAAYWSNMGN
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        GARACNWSNT TYATHACNAA YGGNWSNTGY YTNGCNWSNM GNAARACNWS NTTYATGATG
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        GCNGTNATHG AYGARYTNAT GCARGCNYTN AAYTTYAAYW SNGARACNGT NCCNCARAAR
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      YTNCARYTNA ARCCNYTNAA RAAYWSNMGN CARGTNGARG TNWSNTGGGA RTAYCCNGAY
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A. CLASS	SIFICATION OF SUBJECT MATTER		<del></del>	
IPC 6	C12N15/21 C07K14/56 C12	N15/88 N5/10	C12N15/24 //A61K9/127	C07K14/54
According t	to international Patent Classification (IPC) or to both national	classification an	M IPC	
B. FIELDS	SEARCHED			
Minimum a IPC 6	ocumentation searched (classification system followed by cla CO7K A61K C12N	assification symb	ools)	
	ation searched other than minimum documentation to the exte			
	Jata pase consulted during the international search (name of	data base and	where practical search te	rms used)
	ENTS CONSIDERED TO BE RELEVANT			
Category ·	Citation of document, with indication, where appropriate, o	of the relevant pa	issages	Relevant to claim No.
Y	WO 96 17063 A (VICAL INC) 6 June 1996 (1996-06-06)		÷	1-10, 22-30, 33-36, 42-45, 49-66,68
	the whole document especially page 46, line 1 - line 37; cl	/  aims; fi	gure	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,
		-/		
X Funn	ner documents are listed in the continuation of box C.	X	Patent family members a	re listed in annex.
*Special categories of cited documents:  "A" document defining the general state of the art which is not considered to be of particular relevance  "E" earlier document but published on or after the international filling date  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  "O" document reterring to an oral disclosure, use, exhibition or other means  "P" document published prior to the international filling date but later than the priority date claimed		"X" docu	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention.  "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone.  "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.  "A" document member of the same patent family.  Date of mailing of the international search report.	
	ailing address of the ISA  European Patent Office. P.B. 5818 Patentlaan 2  NL - 2280 HV Rijswijk  Tel (+31-70) 340-2040. Tx 31 651 epo nt.  Fax: (+31-70) 340-3016	Autr	21/10/1999  porized officer  Le Cornec, N	

tntr-national Application No

PCI/US 99/USS94		
C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT  Category   Citation of document, with indication, where appropriate, of the relevant passages   Relevant to claim No.		
	BELLDEGRUN A ET AL: "HUMAN RENAL CARCINOMA LINE TRANSFECTED WITH INTERLEUKIN-2 AND/OR INTERFERON ALPHA GENE(S): IMPLICATIONS FOR LIVE CANCER VACCINES"  JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 85, no. 3, 3 February 1993 (1993-02-03), pages 207-216. XP002057839  ISSN: 0027-8874 cited in the application the whole document	1-10, 22-30, 33-36, 42-45, 49-66,68
Y	RUSSELL J. MUMPER ET AL: "Polyvinyl derivatives as novel interactive polymers for controlled Gene delivery to muscle" PHARMACEUTICAL RESEARCH, vol. 13, no. 5, May 1996 (1996-05), pages 701-709, XP002118167 the whole document	1-10, 22-30, 33-36, 42-45, 49-66,68
Y	H. ALILA ET AL: "Expression of biologically active human Insulin-like growth factor-I following intramuscular injection of a formulated plasmid in rats" HUMAN GENE THERAPY, vol. 8, no. 15, 10 October 1997 (1997-10-10), pages 1785-1795, XP002118452 cited in the application the whole document	1-10, 22-30, 33-36, 42-45, 49-66,68
Y	WO 97 33998 A (CHIOU HENRY C ;CARLO DENNIS J (US); IMMUNE RESPONSE CORP INC (US)) 18 September 1997 (1997-09-18)  page 16, line 30 - line 35; example 1	1-10, 22-30, 42-45, 49-66,68
Y	R.J. MUMPER ET AL: "protective interactive noncondensing (PINC) polymers for enhanced plasmid distribution and expression in rat skeletal muscle" JOURNAL OF CONTROLLED RELEASE, vol. 52, 2 March 1998 (1998-03-02), pages 191-203, XP004113667 the whole document	1-10, 22-30, 33-36, 42-45, 49-66,68

International Application No
Pui/US 99/05394

C.(Continu	ation) DOCUMENTS CONSIDERED TO BE RELEVANT	Pc1/US 99/05394
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
		THE VALUE OF CHAIN NO.
Y	FERRANTINI M ET AL: "ALPHA1-INTERFERON GENE TRANSFER INTO METASTATIC FRIEND LEUKEMIA CELLS ABROGATED TUMORIGENICITY IN IMMUNOCOMPETENT MICE: ANTITUMOR THERAPY BY MEANS OF INTERFERON-PRODUCING CELLS" CANCER RESEARCH, vol. 53, 1 March 1993 (1993-03-01), pages 1107-1112, XP002015124 ISSN: 0008-5472 cited in the application the whole document	1-10, 22-30, 33-36, 42-45, 49-66,68
Y	WO 97 00085 A (UNIV MEDICINE & DENTISTRY OF N) 3 January 1997 (1997-01-03) cited in the application  page 41 ~page 52; claims; example 2	1-10, 22-30, 33-36, 42-45, 49-66,68
Y	WO 96 21470 A (ROLLAND ALAIN ;GENEMEDICINE INC (US); MUMPER RUSSELL J (US)) 18 July 1996 (1996-07-18) cited in the application page 14 -page 15. line 10; claims; examples 1,4	1-10, 22-30, 33-36, 42-45, 49-66,68
A	WO 97 00321 A (WOOD PAUL ;SEOW HENG FONG (AU); COMMW SCIENT IND RES ORG (AU)) 3 January 1997 (1997-01-03) page 31, line 11 -page 32, line 7; claims	11-21
4	M. FERRANTINI ET AL: "IFN-alphal gene expression into a metastatic murine adenocarcinoma (TS/A) results in CD8+ T cell-mediated tumor rejection and development of antitumor immunity" JOURNAL OF IMMUNOLOGY, vol. 153, 1994, pages 4604-4615, XP002118168 the whole document	1-69
	GAO X ET AL: "CATIONIC LIPOSOME-MEDIATED GENE TRANSFER" GENE THERAPY, vol. 2, no. 10, 1 December 1995 (1995-12-01), pages 710-722, XP000749400 ISSN: 0969-7128	
	(continuation of second sheet) (July 1992)	

Pul/US 99/05394

(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT			
legory	Citation of document, with indication, where appropriate, of the relevant passages		
, X	M. COLEMAN ET AL: "Nonviral Interferon alpha Gene therapy inhibits growth of established tumors by eliciting a systemic immune response" HUMAN GENE THERAPY, vol. 9, 10 October 1998 (1998-10-10), pages 2223-2230, XP002118169	1-8, 22-30, 43-45, 50-65	
, А	the whole document	40,42, 46,47, 49,66-69	
°,Х	WO 98 34952 A (GENEMEDICINE INC) 13 August 1998 (1998-08-13)	1-8, 22-40, 42-46, 49-65, 68,69	
	The whole document especially page 11, line 8 - line 33; claims; figure 2	-	
	page 47, line 1 -page 49, line 23		
P,X	WO 98 17689 A (DESHPANDE DEEPA ;FREIMARK BRUCE (US); NORDSTROM JEFF (US); GENEMED) 30 April 1998 (1998-04-30) cited in the application	1-8, 30-39, 42,43, 46, 49-62, 65,68,69	
P,A	the whole document	11-21, 40,41, 44,45, 47,48, 63,66,67	
Т	SK MENDIRATTA ET AL: "Intratumoral delivery of iL-12 gene by polyvinyl polymeric vector system to murine renal and colon carcinoma results in potent antitumor immunity"  GENE THERAPY, vol. 6, no. 5, May 1999 (1999-05), pages 83-839, XP002118170		
	SK MENDIRATTA ET AL: "Intratumoral delivery of iL-12 gene by polyvinyl polymeric vector system to murine renal and colon carcinoma results in potent antitumor immunity"  GENE THERAPY, vol. 6, no. 5, May 1999 (1999-05), pages	11-21 40,41 44,45 47,48	

'ernational application No.

PCT/US 99/05394

Box I	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)
	ernational Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:
1. X	Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  Remark: Although claims 50-55, 59-61, 63-69  are directed to a method of treatment of the human/animal body, (rule 39.1 (IV) PCT, the search has been carried out and based on the alleged effects of the compound/composition.
2	Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
	Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).
Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)
This Inter	national Searching Authority found multiple inventions in this international application, as follows:
1	As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2	As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. A	as only some of the required additional search fees were timely paid by the applicant, this International Search Report sovers only those claims for which fees were paid, specifically claims Nos.;
4. N	lo required additional search fees were timely paid by the applicant. Consequently, this International Search Report is estricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Remark or	The additional search fees were accompanied by the applicant's protest.  No protest accompanied the payment of additional search fees.

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